

## Arabino mycolates from synthetic mycolic acids

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### Tetrahedron

DOI:

[10.1016/j.tet.2016.03.083](https://doi.org/10.1016/j.tet.2016.03.083)

Published: 02/06/2016

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

*Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):*

Mohammed, M. O., Baird, M., Al-Dulayymi, J., Jones, A., & Gwenin, C. (2016). Arabino mycolates from synthetic mycolic acids. *Tetrahedron*, 72(22), 2849-2857. <https://doi.org/10.1016/j.tet.2016.03.083>

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Manuscript Number: TET-D-16-00127R1

Title: Arabino Mycolates from Synthetic Mycolic Acids

Article Type: Full Length Article

Keywords: Mycobacteria  
Tuberculosis  
Arabino mycolates  
Antigens

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Abstract: The synthesis of single mono-arabino mycolates, important lipid antigens from Mycobacteria is described, using structurally defined synthetic mycolic acids. Preliminary assays indicate that these are differentially antigenic to antibodies in the serum of TB-positive patients.

Please find attached a manuscript entitled ‘Arabino Mycolates from Synthetic Mycolic Acids’. Much is known about trehalose and glucose esters of mycolic acids, but there is relatively little in the literature on simple arabinose mycolates, even though penta-arabinose tetramycolates are major components of the cell walls of mycobacteria. We report here the first synthesis of arabino mycolates based on unique synthetic mycolic acids and some preliminary data which show that these molecules are of some potential in the diagnosis of TB.

Please find attached a manuscript modified in line with all the valuable comments made by the reviewer.

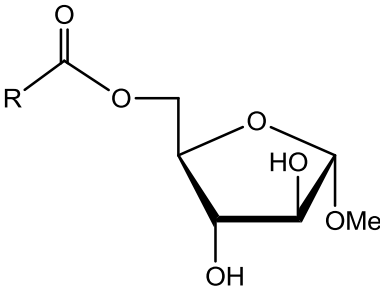
Two accurate mass results have been corrected; our apologies for the errors. Definitions of a number of abbreviations have been included.

# Graphical Abstract

## Arabino Mycolates from Synthetic Mycolic Acids

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R = synthetic mycolic acid



**Tetrahedron**  
journal homepage: [www.elsevier.com](http://www.elsevier.com)



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### ARTICLE INFO

### ABSTRACT

#### Article history:

Received

Received in revised form

Accepted

Available online

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#### Keywords:

*Mycobacteria*

Tuberculosis

Arabino mycolates

Antigens

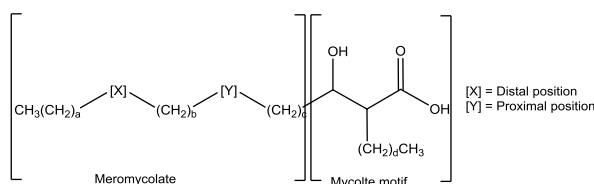
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## 1. Introduction

The mycobacterial cell wall has a complex structure made up of lipids, glycolipids, polysaccharides and proteins.<sup>1</sup> There are four major components: peptidoglycan (PG), mycolyl-arabinogalactan (mAG), lipoarabinomannan (LAM) and extractable lipids.<sup>2</sup> The mycolyl-arabinogalactan (mAG) complex is the largest component structure and acts as a permeability barrier that prevents passage of antibiotics. It forms from cross bonding between both D-arabinofuranosyl (Araf) and-galactofuranosyl (Galf) with a long chain (C70-C90),  $\alpha$ -alkyl branched  $\beta$ -hydroxylated fatty acid, 'mycolic acid'; carbohydrate (Araf) and (Galf) are bound to peptidoglycan in the cell wall by an  $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy- $\alpha$ -D-glucopyranosyl phosphate disaccharide. The galactan part is linear and composed of alternating  $\beta$ -(1 $\rightarrow$ 5) and  $\beta$ -(1 $\rightarrow$ 6) galactofuran residues. Galactan and arabinan are bonded from C-5 of the  $\beta$ -(1 $\rightarrow$ 6) link.<sup>3-6</sup> Both galactosyl and arabinosyl units in mAG are in a furanose form, less stable thermodynamically than the pyranose form.<sup>7</sup> It is believed that this plays a large role in raising the flexibility of the polysaccharide and making the mycolic acids (MAs) pack strongly by van de Waals interaction. Thereby, the structure of the cell wall has extremely low permeability and this provides the organism with high protection from drugs and from its environment.<sup>1</sup> Therefore, studying and understanding mAG biosynthesis is an important strategy for developing new anti-TB drugs. Indeed, isoniazid and ethambutol, two of the standard antibiotics, target mAG biosynthesis; ethambutol inhibits arabinosyltransferases which contribute to the biosynthesis of the arabinan part of the polysaccharides while isoniazid inhibits mycolic acid biosynthesis.<sup>8</sup>

It is approaching a century since Anderson began his ground breaking studies of the lipid components of mycobacteria, leading eventually to the characterization of MAs. These were shown to be high-molecular weight hydroxy acids, with an experimental formula of  $C_{88}H_{172}O_4$  or  $C_{88}H_{176}O_4$  which were very hard to purify and not possible to crystallize.<sup>9,10</sup> The long alkyl branch on the  $\alpha$ -position and hydroxyl groups in  $\beta$ -position in MAs was proved by Asselineau in 1950.<sup>11</sup> Now over 500 related chemical structures of MAs isolated from *Mycobacterium tuberculosis* have been recognized.<sup>12</sup> In brief, MAs can be defined as a complex group of long chain fatty  $\alpha$ -alkyl  $\beta$ -alkyl hydroxyl fatty acids (Figure 1).<sup>13</sup>



**Figure 1:** Generalised mycolic acid structure

The proximal group Y is generally a *cis*-alkene or cyclopropane, or a *trans*-alkene or cyclopropane with a further methyl substituent on the adjacent carbon distal from the hydroxyl acid. The distal group X is normally a *cis*-cyclopropane ( $\alpha$ -MAs), a  $-(CHMeCHOMe)-$  (methoxy MAs) or a  $-(CHMeCO)-$  fragment (keto MAs). Mixtures extracted from mycobacteria comprise many individual MA containing a range of functional groups X and Y (Figure 1) and but each also of several chain lengths.<sup>14</sup>

Anderson & Geiger in 1937 claimed the first extraction of arabino-mycolate from the cell wall of *Mycobacterium bovis*

using natural organic solvent.<sup>15</sup> Some fifty years ago, the isolation of arabinose 5-mycolate by extraction of the cell walls of various mycobacteria under acidic conditions was reported.<sup>16-22</sup> More recently, mass spectrometry and NMR have provided powerful tools for the analysis of such molecules.<sup>23</sup> Azuma and Yamamura in 1962 isolated arabinomycolate as a D-arabinose-5-mycolate and proved it was toxic to mice.<sup>17</sup> Inflammatory reactions similar to that observed after inoculation of live BCG were induced in the lungs by TMM, TDM or GMM isolated from BCG. However, the toxic reactions caused by GroMM and arabinose mono-mycolate were characterized by an acute inflammatory process.<sup>24</sup>

In 2005, the preparation of a tetramycolyl pentaarabinose using a complex natural mixture of MAs was described.<sup>25</sup> However, only in 2010 were structural studies of the composition of the arabinose mycolates of the cell wall of *M. bovis* reported. A two-layer acid hydrolysis gave a number of fractions. One of these was a penta-arabinose tetramycolate, one was an arabinose mono-mycolate, while the others were hexa-arabinose, hepta-arabinose and octa-arabinose tetramycolates. The mycolic acid methyl esters released from each of these showed a mass spectrometric pattern almost identical to the methyl esters obtained by hydrolysis of the original cell wall. A comparison of the penta-arabinose tetramycolate and arabinose monomycolate with samples prepared by combining a mixture of natural MAs with arabinose was reported.<sup>26</sup> Ishiwata *et al* reported the synthesis of a series of mono-, di- and tetra-arabinomycolates found in the terminal position of cell wall skeleton of bacillus Calmette Guerin from *M. bovis*, by using natural mycolic acid mixtures extracted from the cell wall. They proved the biological activity for synthesized compounds in a tumor necrosis factor alpha (TNF- $\alpha$ ) secretion-inducing assay; all the compounds showed strong TNF- $\alpha$  inducing activity *in vitro*.<sup>25</sup> The mechanism of the activity of arabino-mycolate is not clear.<sup>22</sup> Synthetic arabino-mycolates induce the production of TNF- $\alpha$  in murine macrophage cell lines at an intensity similar to BCG-CWS. However the immunological activity of natural arabino-mycolates isolated from BCG has not been investigated, probably due to the complexity of the molecule. Arabino-mycolates obtained by acid hydrolysis from CWS (SMP-105) of *M. bovis* BCG Tokyo 172 strain consisted mainly of mono-arabinose mono-mycolate, pentaarabinose tetra-mycolate and hexa-arabinose tetramycolate fractions. Arabino-mycolates significantly induced TNF- $\alpha$  production with an intensity comparable to that of CWS and enhanced delayed type hypersensitivity (DTH) reactions against inactivated tumor cells. Arabino-mycolates-induced TNF- $\alpha$  production was completely dependent on TLR2 and MyD88 pathways. Thus isolated natural arabino-mycolates possess potent adjuvant immunostimulatory activity.<sup>27</sup>

Intra-tumor injections of extracts of Re mutant *Salmonella typhimurium* in combination with TDM or arabinose mycolate were highly effective in producing regression of tumors in guinea pigs. Similar extracts from *M. bovis* strain BCG and strain AN5 in combination with TDM also possessed tumor-regressive activity. The activity was reduced when the arabinose mycolate was substituted for the TDM. An extract of *Coxiella burnetii*, in combination with either TDM or arabinose mycolate was also active. Intracutaneous administration of Re glycolipid or aqueous extracts from BCG in combination with trehalose or arabinose mycolates did not produce life-threatening, clinical signs of toxicity in young mice. If additional toxicity studies demonstrate that adverse side effects can be satisfactorily controlled, these water soluble extracts may prove beneficial in the treatment of spontaneous tumors of humans and other animals.<sup>28,29</sup> The

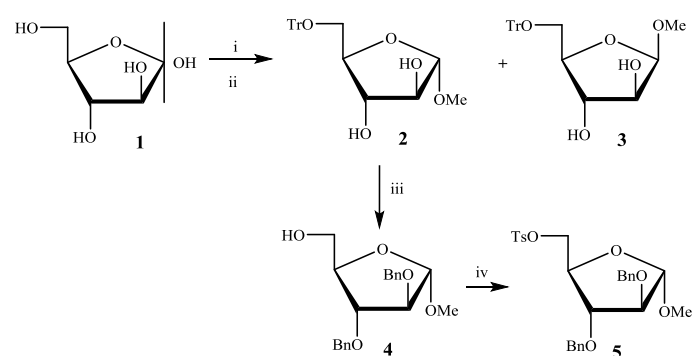
adjuvant activity of cell wall skeletons (mycolic acid-arabino-galactan-mucopeptide, CWS) prepared from the cells of mycobacteria, nocardia and corynebacteria was examined in vivo in mice and guinea pigs. The cell wall skeletons of *M. bovis* BCG (BCG-CWS), *Nocardia asteroides* 131 and *Corynebacterium diphtheriae* PWC suspended in Freund's incomplete adjuvant (FIA) as water-in-oil emulsions showed potent adjuvant activity on the formation of circulating antibody and cell-mediated immunity to bovine serum albumin (BSA), sheep erythrocytes (SRBC) and sulfanyazo-bovine serum albumin (SA-BSA) in mice and guinea pigs. After acetylation or acid treatment, BCG-CWS retained its adjuvant activity, but the activity of BCG-CWS was destroyed completely by alkaline treatment. The cell wall constituents, arabinose-mycolate and arabino-galactan, prepared from BCG-CWS showed no adjuvant activity.<sup>30</sup>

Few studies of arabinose mycolates have been carried out and little is known of the effect of structure on the immunostimulatory activity of arabino-mycolates in activating macrophages.<sup>6</sup> However, D-arabinose-5-mycolate, purified from bound lipids of the cell-wall skeleton of *M. bovis* BCG may be prominent structure for recognition by host immunity.<sup>6</sup>

However, it is now clearly established that, in the case of free MAs, the detailed structure controls their effects on a range of cytokines and chemokines and that some lead to inflammatory reactions on intratracheal instillation in mice, while others lead to the formation of foam cells.<sup>31</sup> we now report the synthesis of a set of arabinose esters of stereochemically defined synthetic MAs, as well as those of some simple fatty acids.

## 2a Results and discussion: synthesis of arabinose mycolates

According to literature procedures, D-(-)-arabinose **1** was treated with HCl (0.22 N) freshly prepared by addition of acetyl chloride to anhydrous methanol at 0 °C working up with pyridine rather than ammonium carbonate,<sup>32</sup> to give methyl- $\alpha,\beta$ -D-arabino furanoside with predominant formation of the  $\alpha$ -anomer ( $\alpha$ -D/ $\beta$ -D 3:2).<sup>33,34</sup> In order to isolate these two anomers, *in situ* tritylation of this mixture followed by column chromatography gave methyl 5-O-trityl  $\alpha$ -D-Araf **2** (45%) and 5-O-trityl  $\beta$ -D-Araf **3** (30%). Compound **2** was benzylated to protect the two secondary hydroxyl groups by using benzyl bromide and sodium hydride in dry DMF then the trityl group was removed from the primary hydroxyl group by hydrolysis in 80% AcOH, affording compound **4** (Scheme 1).<sup>25,34</sup>



**Scheme 1.** Reagents and conditions: (i) HCl, CH<sub>3</sub>OH; (ii) Trityl chloride, DMAP, pyridine, 0 °C/R.T., then 70 °C 4 h, 45% from  $\alpha$ -anomer; (iii) a: NaH, BnBr, DMF, 0 °C/R.T. 2 h, b: 80% AcOH, 75 °C for 4 h, 78%; (iv) 4-toluenesulfonyl chloride, DMAP, pyridine, 90%.

According to the literature,<sup>25</sup> direct esterification between the primary hydroxyl group of Araf and a carboxyl group in natural mycolic acid mixtures was achieved in a low yield (30%), while activating the sugar as a tosylate raised the yield to 79%. Therefore, the hydroxyl group in the compound **4** was tosylated by reaction with p-toluenesulfonyl chloride in dry pyridine and catalytic 4-dimethylaminopyridine (DMAP) in dry CH<sub>2</sub>Cl<sub>2</sub> at 0 °C to afford the tosylate **5**. Surprisingly, esters of arabinose with simple fatty acids do not appear to have been reported. In a model experiment, compound **4** was therefore first condensed with behenic acid using DMAP and N,N-dicyclohexylcarbodiimide in dry CH<sub>2</sub>Cl<sub>2</sub> to give compound **7a** in 80% yield.<sup>36</sup> This was then hydrogenolysed in the presence of Pd(OH)<sub>2</sub> and under a hydrogen atmosphere to give the behenyl arabinose **8a**. Compound **5** was also reacted with palmitic acid by an alkylative esterification using cesium hydrogen carbonate in dry DMF: THF at 70 °C,<sup>25</sup> to give compound **7b**; this was then debenzylated to give the compound **8b**. In the same way, compound **5** was reacted with oleic acid to give the compound **7c**; hydrogenolysis in the presence of Pd(OH)<sub>2</sub> under a hydrogen atmosphere gave the corresponding stearyl arabinose, in which the double bond was also saturated. However, deprotection with boron trichloride in dichloromethane and methanol gave the oleoyl derivative **8c**.

A series of synthetic MAs was then reacted with the compound **5** by alkylative esterification using cesium hydrogen carbonate as in **Scheme 2**. Firstly, the methoxy *cis*-cyclopropane mycolic acid **6d**,<sup>37</sup> gave compound **7d**, which was then debenzylated using hydrogen and Pd(OH)<sub>2</sub> catalyst to give the arabinose ester **8d**. This was achieved with no loss of the cyclopropane group, on the basis of the proton and carbon NMR spectra. Although the absolute stereochemistry of the *cis*-cyclopropane unit in MAs remains unproven, this compound contains the stereochemistry that appears likely, based on a common intermediate leading to the different classes of mycolic acid.<sup>38</sup> The 22 carbon length of the  $\alpha$ -chain is typical of mycobacteria such as *Mycobacterium kansasii*.<sup>39</sup> Condensation of methoxy-*cis*-cyclopropane mycolic acid **6e**,<sup>37</sup> having a twenty two carbon chain in the  $\alpha$ -position, and the opposite absolute *cis*-cyclopropane stereochemistry, one stereoisomer of which is present in *M. kansasii*, led to compound **7e**. On hydrogenolysis as above, this gave compound **8e**. In the same way, esterification of methoxy-*cis*-cyclopropane mycolic acid **6f**,<sup>40</sup> with a twenty four carbon chain at the  $\alpha$ -position, a stereoisomer of which is a major component of *M. tuberculosis* methoxy-MA,<sup>39</sup> but the opposite cyclopropane stereochemistry to **6d** gave compound **7f**, which on hydrogenolysis led to compound **8f**.

The same procedure was repeated to prepare the arabino ester compounds for the keto *cis*-cyclopropane mycolic acid **6g**,<sup>37</sup> with a C<sub>22</sub> alkyl chain in the  $\alpha$ -position; this was used as the epimeric mixture at the carbon adjacent to the ketone. This produced compound **7g** which on debenzylation gave the arabinose ester **8g**. Ester **8g'**, with the normal C<sub>24</sub> *M. tuberculosis*  $\alpha$ -chain length, was also obtained from **6g'**.<sup>41</sup> In the same way the arabinose ester **8h**, from the keto *trans*-cyclopropane mycolic acid **6h**,<sup>42</sup> again epimeric adjacent to the ketone, was obtained in good yield. Finally, the  $\alpha$ -mycolic acid **6i**,<sup>43,44</sup> was esterified to give compound **7i** and then debenzylation to give **8i**.

The yields for each of these coupling steps and deprotections are given in **Table 1**. All the products were characterized by accurate mass MALDI-MS, proton and carbon NMR and the spectra are presented as Supplementary Information.





This shows that the six synthetic arabinose mycolates are detected to a different degree by antibodies in the serum of patients infected with active tuberculosis, and that in some cases the response is no different between TB+ and TB- samples. The TB status had been determined using a range of standard assays employed by the WHO, including being smear and culture positive; the TB- samples were all from patients showing some of the symptoms of TB, but diagnosed using the same methods as not having active TB, and being both smear and culture negative. Although this is a very small sample set, it is interesting that the highest distinction between TB+/TB- is given with the arabinose mycolates **7d** and **7i** which probably represent the natural stereochemistries of methoxy- and alpha-mycolates. Work is ongoing to validate these results on a larger sample set.

The effects of selected examples of the above synthetic arabinose mono-mycolates on the Mincle receptor in *M. tuberculosis* will be described elsewhere.<sup>47</sup>

### 3. Experimental section

Chemicals used were obtained from commercial suppliers (Sigma, Aldrich, and Alfa Aesar) or prepared from them by the methods described. Solvents which were required to be dry, e.g. ether, tetrahydrofuran were dried over sodium wire and benzophenone under nitrogen, while dichloromethane and HMPA were dried over calcium hydride. All reagents and solvents used were of reagent grade unless otherwise stated. Organic solutions were dried over anhydrous magnesium sulfate. Silica gel (Merck 7736) and silica gel plates used for column chromatography and thin layer chromatography were obtained from Aldrich; separated components were detected using variously UV light, I<sub>2</sub> and phosphomolybdic acid solution in IMS followed by charring. Infra-red (IR) spectra were carried out on a Perkin-Elmer 1600 F.T.I.R. spectrometer as liquid films or KBr disc (solid). Melting points were measured using a Gallenkamp melting point apparatus. NMR spectra were carried out on a Bruker AC250 or Advance 400 spectrometer.  $[\alpha]_D$  values were recorded in CHCl<sub>3</sub> on a POLAAR 2001 optical activity polarimeter. Mass spectra were recorded on a Bruker matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) values are given plus sodium.

#### 3.1: Methyl 5-O-trityl- $\alpha$ -D-Araf 2

Freshly prepared HCl solution in MeOH (resulting from mixing acetyl chloride (2 mL) in MeOH (30 mL) at 0 °C) was added to a stirred solution of D-(-)-arabinose (5.0 g, 33 mmol) in anhydrous MeOH (100 mL). Stirring was continued overnight at room temperature, when a clear solution was obtained. The mixture was neutralized by adding pyridine to pH 7 – 8, and the solid was filtered and washed with MeOH (10 mL). The solvent was evaporated to give a residue which was purified by column chromatography eluting with chloroform: acetone (3:5) to give a colourless oil, methyl- $\alpha,\beta$ -D-Araf (4.3 g, 78%). Trityl chloride (6.42 g, 23.0 mmol) and 4-(dimethylamino)pyridine (2.57 g, 21.0 mmol) were added to a stirred solution of methyl- $\alpha,\beta$ -D-Araf (3.12 g, 19.0 mmol) in anhydrous pyridine (60 mL) and the mixture was stirred at room temperature overnight then heated on oil bath at 70 °C for 4 h. The mixture was cooled to room temperature and poured into ice/water (300 mL). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3  $\times$  100 mL). The combined organic layers were washed with aq. NaHCO<sub>3</sub> solution (5%, 100 mL), dried and the solvent was evaporated. The residue was purified by column chromatography eluting started from 10% to 50% hexane/ethyl

acetate gave the title compound as a colorless oil **2** (3.3 g, 45%) and 5-O-trityl- $\beta$ -D-Araf **3** (2.3 g, 30%). The mixture of anomers showed  $\delta_H$  (400 MHz, CDCl<sub>3</sub>),  $\delta_C$  (125 MHz, CDCl<sub>3</sub>) and  $\nu_{max}$  identical to the literature.<sup>33,34</sup>

#### 3.2: Methyl 2,3-di-O-benzyl- $\alpha$ -D-Araf 4

A solution of **2** (3.20 g, 7.88 mmol) in dry DMF (80 mL) was added dropwise to a stirred suspension of NaH (0.85 g) (60% w/w, dispersion in mineral oil, washed with petrol three times) at room temperature under nitrogen. The mixture was stirred for 30 min. then benzyl bromide (2.5 mL, 3.6 g, 21.0 mmol) in dry DMF (50 mL) was added. The mixture was stirred at room temperature for 20 h and then quenched by slow addition of water (15 mL) and diluted with ether (25 mL). The organic layer was separated and the aqueous layer was extracted with ether (2  $\times$  100 mL). The combined extracts were washed with water (50 mL), brine (50 mL), dried and evaporated under reduced pressure. Aqueous acetic acid (80%, 30 mL) was added to the crude product and the mixture was stirred and heated at 75 °C for 4 h, then diluted with water (20 mL) and ether (20 mL) and the organic layer was separated and the aqueous layer extracted with ether (2  $\times$  100 mL). The combined organic layers were washed with water (50 mL), sat. aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL), dried and the solvent was evaporated. Column chromatography eluting with petrol/ethyl acetate (7:3) gave the title compound as a colorless oil **4** (2.12 g, 78%) [MALDI-Found (M+Na)<sup>+</sup>: 367.3; C<sub>20</sub>H<sub>24</sub>NaO<sub>5</sub>, requires: 367.1],  $[\alpha]_D^{22} +89$  (c 0.10, CHCl<sub>3</sub>) [lit.<sup>26</sup>  $[\alpha]_D^{24} +83.2$  (c 1.14, CHCl<sub>3</sub>)], which showed  $\delta_H$  (400 MHz, CDCl<sub>3</sub>): 7.40 – 7.28 (10H, m), 4.95 (1H, s), 4.61 (1H, d, *J* 12.0 Hz), 4.54 (1H, d, *J* 12 Hz), 4.53 (1H, d, *J* 12 Hz), 4.50 (1H, d, *J* 12 Hz), 4.18 – 4.12 (1H, m), 4.02 – 3.96 (2H, m), 3.85 (1H, dd, *J* 12.1, 2.8 Hz), 3.65 (1H, dd, *J* 12.1, 4.1 Hz), 3.40 (3H, s), 2.93 (1H, br. s);  $\delta_C$  (101 MHz, CDCl<sub>3</sub>): 137.6, 137.2, 128.4, 128.4, 127.9, 127.8, 127.79, 127.7, 107.3, 87.6, 82.5, 82.2, 72.3, 71.8, 62.1, 54.8;  $\nu_{max}$ : 3466 br., 3089, 3064, 3031, 2925, 1725, 1605, 1454, 739 cm<sup>-1</sup>. All data were identical to the literature.<sup>25,34</sup>

#### 3.3: Methyl 2,3-di-O-benzyl-5-O-p-toluensulfonyl- $\alpha$ -D-Araf 5

*p*-Toluenesulfonyl chloride (0.60 g, 3.14 mmol) was added to a stirred solution of **4** (0.50 g, 1.45 mmol), pyridine (0.96 g, 0.98 mL, 12.4 mmol) and DMAP (0.10 g, 0.81 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (5 mL) at 0 °C. The mixture was stirred at room temperature for 16 h then diluted with ethyl acetate (10 mL), the organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2  $\times$  100 mL). The combined organic layer were washed with water (50 mL), brine (50 mL), dried and the solvent was evaporated. The residue was purified by column chromatography eluting with hexane/ethyl acetate (4:1) affording the title compound as a colorless oil **5** (0.65 g, 90%),  $[\alpha]_D^{23} +55$  (c 0.10, CHCl<sub>3</sub>) [lit.<sup>25</sup>  $[\alpha]_D^{27} +57.0$  (c 0.40, CHCl<sub>3</sub>)], which showed  $\delta_H$  (400 MHz, CDCl<sub>3</sub>): 7.77 (2H, d, *J* 8.2 Hz), 7.41 – 7.25 (12H, m), 4.85 (1H, s), 4.56 – 4.38 (4H, m), 4.22 – 4.15 (1H, m), 4.12 (2H, br d, *J* 4.6 Hz), 3.94 (1H, br d, *J* 2.6 Hz), 3.81 (1H, dd, *J* 5.9, 2.7 Hz), 3.33 (3H, s), 2.42 (3H, s);  $\delta_C$  (101 MHz, CDCl<sub>3</sub>): 144.8, 137.3, 137.2, 132.7, 129.7, 128.4, 128.0, 127.9, 127.89, 127.8, 127.79, 107.4, 87.5, 82.8, 79.2, 72.2, 71.9, 68.8, 55.1, 21.6;  $\nu_{max}$ : 3064, 3032, 2916, 1741, 1454, 1365, 1177, 698 cm<sup>-1</sup>. All data were identical to the literature.<sup>25</sup>

#### 3.4: Methyl 5-O-behenoyl- $\alpha$ -D-Araf 8a

(a) A solution of N,N'-dicyclohexylcarbodiimide (0.089 g, 0.432 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1 mL) was added dropwise to a stirred solution of **4** (0.10g, 0.29 mmol), DMAP (0.042 g, 0.343 mmol) and behenic acid (0.10 g, 0.29 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1 mL) at 0 °C under nitrogen. The mixture was stirred for 30 min. then the precipitate of dicyclohexyl urea was filtered off and washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), the solvent was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (10:1) to afford *methyl 2,3-di-O-benzyl-5-O-behenoyl-α-D-Araf*<sup>β1</sup> as a colorless oil **7a** (0.15 g, 80%) [MALDI-Found (M+Na)<sup>+</sup>: 689.5, C<sub>42</sub>H<sub>66</sub>NaO<sub>6</sub> requires: 689.4], [α]<sub>D</sub><sup>24</sup> +40 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 7.41 – 7.28 (10H, m), 4.95 (1H, s), 4.59 (1H, d, *J* 12.0 Hz), 4.57 (1H, d, *J* 12 Hz), 4.52 (1H, d, *J* 12 Hz), 4.49 (1H, d, *J* 12 Hz), 4.29 (1H, dd, *J* 11.3, 2.8 Hz), 4.24 – 4.15 (2H, m), 4.01 (1H, dd, *J* 2.9, 1.1 Hz), 3.84 (1H, dd, *J* 6.3, 2.9 Hz), 3.40 (3H, s), 2.33 – 2.26 (2H, m), 1.64 – 1.52 (2H, m), 1.34 – 1.18 (36H, m), 0.89 (3H, t, *J* 6.9 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.6, 137.5, 137.4, 128.45, 128.43, 127.9, 127.8, 107.3, 88.0, 83.4, 79.4, 72.3, 72.0, 63.5, 55.0, 34.1, 31.9, 29.7, 29.6, 29.62, 29.5, 29.3, 29.27, 29.1, 24.8, 22.7, 14.1; ν<sub>max</sub>: 3034, 2915, 2849, 1741, 1471, 1100, 758 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0026 g, 0.15 fold by weight) was added to a stirred solution of **7a** (0.010g, 0.01 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub>: MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The reaction mixture was stirred for 24 h then filtered and the precipitate washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), the filtrate was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (1:1) to afford the title compound as a colorless oil **8a** (0.0079 g, 81%) [MALDI-Found (M+Na)<sup>+</sup>: 509.5, C<sub>28</sub>H<sub>54</sub>NaO<sub>6</sub> requires: 509.3], [α]<sub>D</sub><sup>22</sup> +46 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 4.92 (1H, s), 4.32 – 4.29 (2H, m), 4.22 (1H, dd, *J* 7.1, 3.9 Hz), 4.09 (1H, br. s), 3.90 (1H, br.s), 3.42 (3H, s), 2.81 – 2.67 (1H, m), 2.51 – 2.39 (1H, m), 2.38 – 2.32 (2H, m), 1.62 (2H, dd, *J* 14.7, 7.3 Hz), 1.35 – 1.20 (36H, m), 0.89 (3H, t, *J* 7.0 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.5, 108.8, 83.7, 79.8, 78.0, 63.8, 55.0, 34.1, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 24.8, 22.6, 14.1; ν<sub>max</sub>: br. 3500, 2917, 2850, 1739, 1464, 1100, 758 cm<sup>-1</sup>.

### 3.5: Methyl 2,3-di-O-benzyl-5-O-palmitoyl-α-D-Araf **8b**

(a) Cesium hydrogen carbonate (0.19 g, 1.0 mmol) was added to a stirred solution of **5** (0.1 g, 0.2 mmol) and palmitic acid (0.061 g, 0.237 mmol) in dry DMF:THF (1: 5, 5 mL) at room temperature and the reaction mixture was stirred at 70 °C for two days. The suspension was diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL), dried, filtered and evaporated to give a thick oil residue. The residue was purified by column chromatography eluting with hexane/ethyl acetate (10:1) to give *methyl 2,3-di-O-benzyl-5-O-palmitoyl-α-D-Araf* **7b** (0.089 g, 76%)<sup>25</sup> as a colorless oil [MALDI-Found (M+Na)<sup>+</sup>: 605.1, C<sub>36</sub>H<sub>54</sub>NaO<sub>6</sub> requires: 605.3], [α]<sub>D</sub><sup>22</sup> +37 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.95 (1H, s), 4.59 (1H, d, *J* 12.0 Hz), 4.57 (1H, d, *J* 11.7 Hz), 4.52 (1H, d, *J* 12.0 Hz), 4.49 (1H, d, *J* 11.8 Hz), 4.29 (1H, dd, *J* 11.3, 2.9 Hz), 4.24 – 4.15 (2H, m), 4.01 (1H, dd, *J* 3.0, 1.2 Hz), 3.84 (1H, dd, *J* 6.2, 2.9 Hz), 3.40 (3H, s), 2.32 – 2.27 (2H, m), 1.64 – 1.54 (2H, m), 1.26 (24H, s), 0.89 (3H, t, *J* 7.0 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.6, 137.5, 137.3, 128.4, 128.3, 127.9, 127.8, 107.3, 88.0, 83.4, 79.3, 72.3, 72.0, 63.5, 54.9, 34.1, 31.9, 29.7, 29.68, 29.6, 29.4, 29.32,

29.3, 29.1, 24.8, 22.7, 14.1; ν<sub>max</sub>: 3064, 3032, 2924, 2853, 1740, 1455, 1365, 1107, 735 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.01 g, 0.15 fold by weight) was added to a stirred solution of **7b** (0.06 g, 0.10 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub>: MeOH, (1:1, 2 mL) at room temperature under hydrogen atmosphere. The reaction mixture was stirred for 24 h then filtered and the precipitate was washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), the filtrate was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (1:1) to afford the title compound as a colorless oil **8b** (0.04 g, 88%),<sup>34</sup> [α]<sub>D</sub><sup>18</sup> +1.2 (c 0.10, CHCl<sub>3</sub>) [MALDI-Found (M+Na)<sup>+</sup>: 425.5, C<sub>22</sub>H<sub>42</sub>NaO<sub>6</sub> requires: 425.2] which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 4.93 (1H, s), 4.33 – 4.30 (2H, m), 4.22 (1H, dd, *J* 6.9, 3.9 Hz), 4.09 (1H, br. s), 3.91 (1H, br. s), 3.43 (3H, s), 2.35 (2H, t, *J* 7.6 Hz), 1.67 – 1.60 (2H, m), 1.56 (2H, br. s), 1.36 – 1.21 (24H, m), 0.89 (3H, t, *J* 6.9 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.5, 108.8, 83.8, 79.8, 78.0, 63.8, 55.1, 34.1, 31.9, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 24.8, 22.6, 14.1; ν<sub>max</sub>: br. 3308, 2916, 2848, 1742, 1472, 1099, 728 cm<sup>-1</sup>.

### 3.6: Methyl 5-O-oleoyl-α-D-Araf **8c**

(a) Cesium hydrogen carbonate (0.47 g, 2.42 mmol) was added to a stirred solution of **5** (0.26 g, 0.52 mmol) and oleic acid (0.10 g, 0.35 mmol) in dry DMF:THF (1:5, 2 mL) at room temperature and the mixture was stirred at 70 °C for two days. The suspension was diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL). The organic layer was dried, filtered and evaporated to give a thick oil residue. This was purified by column chromatography eluting with hexane/ethyl acetate (7:2) to afford *methyl 2,3-di-O-benzyl-5-O-oleoyl-α-D-Araf* as a colorless oil **7c** (0.18 g, 83%) [MALDI-Found (M+Na)<sup>+</sup>: 631.3, C<sub>38</sub>H<sub>56</sub>NaO<sub>6</sub> requires: 631.4], [α]<sub>D</sub><sup>22</sup> +45 (c 0.10, CHCl<sub>3</sub>) which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 5.41 – 5.30 (2H, m), 4.95 (1H, s), 4.59 (1H, d, *J* 12.0 Hz), 4.57 (1H, d, *J* 12 Hz), 4.52 (1H, d, *J* 12 Hz), 4.49 (1H, d, *J* 12 Hz), 4.29 (1H, dd, *J* 11.0, 2.5 Hz), 4.24 – 4.15 (2H, m), 4.0 (1H, br d, *J* 2.1 Hz), 3.84 (1H, dd, *J* 6.2, 2.8 Hz), 3.40 (3H, s), 2.30 (2H, t, *J* 7.6 Hz), 2.06 – 1.97 (4H, m), 1.64 – 1.54 (4H, m), 1.28 (18H, br m), 0.89 (3H, t, *J* 6.8 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.6, 137.5, 137.4, 130.0, 129.7, 128.5, 128.4, 127.9, 107.3, 87.9, 83.3, 79.3, 72.3, 72.0, 63.5, 55.0, 34.0, 31.9, 29.7, 29.6, 29.5, 29.3, 29.17, 29.11, 29.1, 27.25, 27.2, 24.8, 22.6, 14.1; ν<sub>max</sub>: 3005, 3089, 3031, 2926, 2855, 1740, 1454, 1050, 735 cm<sup>-1</sup>.

(b) Boron trichloride<sup>48</sup> (0.32 mL, 1M) was added to a stirred solution of compound (**7c**) (0.02 g, 0.03 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (2 mL) at -78 °C. The mixture was stirred for 2 h then quenched with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (1:1, 2 mL) and the solvent was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (1:1) to afford the title compound (**7c**) (9 mg, 64%) [Found (MALDI) (M+Na)<sup>+</sup>: 451.3, C<sub>24</sub>H<sub>44</sub>NaO<sub>6</sub> requires: 451.3], [α]<sub>D</sub><sup>18</sup> +5.0 (c 0.10, CHCl<sub>3</sub>) which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub> + few drops CD<sub>3</sub>OD): 5.35 – 5.26 (2H, m), 4.77 (1H, d, *J* 4.3 Hz), 4.24 (1H, dd, *J* 11.7, 2.1 Hz), 4.11 – 4.05 (1H, m), 4.02 – 3.97 (1H, m), 3.97 – 3.91 (2H, m), 3.39 (3H, s), 2.32 (2H, t, *J* 7.6 Hz), 2.04 – 1.92 (4H, m), 1.65 – 1.54 (2H, m), 1.35 – 1.15 (22H, m), 0.84 (3H, t, *J* 6.7 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub> + few drops CD<sub>3</sub>OD): 174.3, 129.96, 129.7, 102.3, 79.9, 77.7, 75.8, 65.4, 55.2, 49.6, 49.3, 49.1, 48.9, 48.7, 34.1, 31.8, 29.7, 29.6, 29.4, 29.2, 29.1, 29.0, 27.1, 27.09, 24.8, 22.6, 13.98; ν<sub>max</sub>: br. 3468, 2917, 2850, 1735, 1454, 1050, 824 cm<sup>-1</sup>.

### 3.7: Methyl 5-O-stearyl- $\alpha$ -D-Araf

Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.012 g, 0.15 fold by weight) was added to a stirred solution of **7c** (0.08 g, 0.13 mmol) in dry (CH<sub>2</sub>Cl<sub>2</sub> : MeOH, 1:1, 3 mL) at room temperature under hydrogen atmosphere. The mixture was stirred for 24 h then filtered and the precipitate washed with CH<sub>2</sub>Cl<sub>2</sub> (15 mL), the filtrate was evaporated and the residue was purified by column chromatography eluting with hexane / ethyl acetate (1:1) to afford the title compound as a colorless oil (0.045 g, 80%) [MALDI-Found (M+Na)<sup>+</sup>: 453.0, C<sub>24</sub>H<sub>46</sub>NaO<sub>6</sub> requires: 453.3]; [ $\alpha$ ]<sub>D</sub><sup>18</sup> +2 (c 0.1, CHCl<sub>3</sub>);  $\delta_H$  (400 MHz, CDCl<sub>3</sub>): 4.92 (1H, s), 4.30 (2H, d, *J* 4.0 Hz), 4.21 (1H, dd, *J* 7.3, 3.8 Hz), 4.17 – 4.06 (1H, m), 3.90 (1H, br d, *J* 10.1 Hz), 3.42 (3H, s), 2.77 (1H, d, *J* 10.2 Hz), 2.52 (1H, d, *J* 7.9 Hz), 2.35 (2H, t, *J* 7.6 Hz), 1.69 – 1.58 (2H, m), 1.34 – 1.21 (28H, m), 0.88 (3H, t, *J* 6.8 Hz);  $\delta_C$  (101 MHz, CDCl<sub>3</sub>): 173.5, 108.8, 83.7, 79.8, 78.0, 63.8, 55.1, 50.8, 34.1, 31.9, 29.7, 29.6, 29.6, 29.4, 29.3, 29.2, 29.05, 24.79, 22.67, 14.10;  $\nu_{max}$ : br. 3468, 2917, 2850, 1735, 1454, 1050, 824 cm<sup>-1</sup>.

### 3.8: Methyl 5-O-(2-[(*R*)-1-hydroxy-18-[(1*S*, 2*R*)-2-[(17*S*, 18*S*)-17-methoxy-18-methylhexatriacontyl]cyclopropyl]octadecyl]tetracosanoate) $\alpha$ -D-Araf **8d**

(a) Cesium hydrogen carbonate (0.056 g, 0.288 mmol) was added to a solution of **5** (0.031g, 0.062 mmol) and (*R*)-2-[(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-[(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl] cyclopropyl]octadecyl]tetracosanoic acid (0.051g, 0.041 mmol)<sup>37</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and the mixture was stirred at 70 °C for two days. Work up as before gave methyl 2,3-di-*O*-benzyl-5-*O*-(2-[(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-[(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl]cyclopropyl]octadecyl]- tetracosanoate)  $\alpha$ -D-Araf **7d** as a thick colorless oil (0.05 g, 77%), [Found (M+Na)<sup>+</sup>: 1574.4044, C<sub>103</sub>H<sub>186</sub>NaO<sub>8</sub> requires: 1574.4040]; [ $\alpha$ ]<sub>D</sub><sup>20</sup> +23 (c 0.10, CHCl<sub>3</sub>);  $\delta_H$  (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12.0 Hz), 4.56 (1H, d, *J* 12 Hz), 4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.33 – 4.27 (2H, m), 4.25 – 4.19 (1H, m), 3.99 (1H, br d, *J* 2.0 Hz), 3.84 (1H, dd, *J* 6.4, 2.6 Hz), 3.67 – 3.59 (1H, m), 3.37 (3H, s), 3.35 (3H, s), 2.99 – 2.94 (1H, m), 2.52 (1H, d, *J* 8.3 Hz), 2.43 (1H, dt, *J* 9.4, 5.4 Hz), 1.72 – 1.61 (2H, m), 1.59 – 1.03 (141H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H, d, *J* 6.9 Hz), 0.70 – 0.61 (2H, m), 0.60 – 0.53 (1H, dt, *J* 4, 8 Hz), –0.33 (1H, br. q *J* 5.1 Hz);  $\delta_C$  (101 MHz, CDCl<sub>3</sub>): 175.0, 128.5, 128.4, 127.9, 127.8, 107.2, 87.9, 85.4, 83.7, 79.4, 72.4, 72.1, 63.5, 57.7, 54.9, 51.53, 35.52, 35.3, 32.36, 31.9, 30.5, 30.48, 30.4, 30.3, 30.23, 30.12, 30.0, 29.98, 29.94, 29.9, 29.86, 29.84, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.26, 29.2, 29.19, 29.1, 29.0, 29.04, 28.7, 28.67, 27.5, 27.4, 26.1, 25.7, 22.7, 22.6, 15.7, 14.8, 14.1, 10.9;  $\nu_{max}$ : 3479, 3064, 2924, 2853, 1735, 1494, 1455, 1100 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0033 g, 0.15 fold by weight) was added to a stirred solution of **7d** (0.022 g, 0.014 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight; work up as before gave the title compound as a thick colorless oil **8d** (0.016 g, 79%) [Found (M+NH<sub>4</sub>)<sup>+</sup>: 1389.3550, C<sub>89</sub>H<sub>178</sub>NO<sub>8</sub> requires: 1389.3552]; [ $\alpha$ ]<sub>D</sub><sup>21</sup> +28 (c 0.10, CHCl<sub>3</sub>);  $\delta_H$  (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.49 (1H, dd, *J* 12, 4.0 Hz), 4.35 (1H, dd, *J* 12, 4.1 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br.s), 3.98 (1H, br.s), 3.77 – 3.66 (1H, m), 3.41 (3H, s),

3.35 (3 H, s), 3.01 – 2.92 (1H, m), 2.85 (1H, s), 2.54 – 2.36 (1H, ddd, *J* 10.1, 6.9, 5.1 Hz), 1.48 – 1.17 (144H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H, d, *J* 6.9 Hz), 0.70 – 0.61 (2H, m), 0.61 – 0.52 (1H, dt, *J* 7.9, 3.9 Hz), –0.33 (1H, br. q *J* 5.1 Hz);  $\delta_C$  (101 MHz, CDCl<sub>3</sub>): 174.9, 108.7, 85.4, 83.6, 80.5, 78.4, 72.8, 63.2, 57.7, 54.9, 52.3, 35.3, 35.2, 32.3, 31.9, 30.7, 30.69, 30.64, 30.62, 30.55, 30.5, 30.4, 30.37, 30.3, 30.28, 30.2, 30.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.57, 29.55, 29.5, 29.4, 29.3, 29.28, 29.25, 29.2, 29.1, 29.0, 29.03, 29.0, 28.9, 28.7, 28.6, 28.5, 28.3, 27.5, 27.4, 26.1, 25.4, 22.6, 15.7, 14.8, 14.1, 10.9;  $\nu_{max}$ : br.3435, 2918, 2850, 1732, 1455, 1100 cm<sup>-1</sup>.

### 3.9: Methyl 5-O-(2-[(*R*)-1-hydroxy-18-[(1*R*, 2*S*)-2-[(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl]cyclo propyl]octadecyl]tetracosanoate) $\alpha$ -D-Araf **8e**

(a) Cesium hydrogen carbonate (0.098 g, 0.505 mmol) was added to a stirred solution of **5** (0.0546 g, 0.1000 mmol) and (*R*)-2-[(*R*)-1-hydroxy-18-[(1*R*,2*S*)-2-[(17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl]cyclopropyl]octadecyl]tetracosanoic acid (0.100 g, 0.081 mmol)<sup>37</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and the reaction mixture was stirred at 70 °C for two days. The suspension was diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL), then dried, filtered and evaporated to give a thick oil residue. The residue was purified by column chromatography eluting with hexane/ethyl acetate (10:1) to give methyl 2,3-di-*O*-benzyl-5-*O*-(2-[(*R*)-1-hydroxy-18-[(1*R*,2*S*)-2-[(17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl]-cyclopropyl]octadecyl]tetracosanoate) $\alpha$ -D-Araf as a colorless thick oil **7e** (0.095 g, 75%) [Found (M+Na)<sup>+</sup>: 1574.4085, C<sub>103</sub>H<sub>186</sub>NaO<sub>8</sub> requires: 1574.4040], [ $\alpha$ ]<sub>D</sub><sup>23</sup> +18 (c 0.10, CHCl<sub>3</sub>), which showed  $\delta_H$  (500 MHz, CDCl<sub>3</sub>): 7.38 – 7.29 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12 Hz), 4.56 (1H, d, *J* 12 Hz), 4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.31 – 4.28 (2H, m), 4.24 – 4.20 (1H, m), 3.99 (1H, dd, *J* 2.7, 0.9 Hz), 3.84 (1H, dd, *J* 6.4, 2.6 Hz), 3.66 – 3.60 (1H, m), 3.38 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.52 (1H, br. s), 2.43 (1H, dt, *J* 9.1, 5.5 Hz), 1.72 – 1.61 (2H, m), 1.60 – 1.07 (141H, m), 0.89 (6H, t, *J* 6.9 Hz), 0.86 (3H, d, *J* 6.8 Hz), 0.69 – 0.62 (2H, m), 0.57 (1H, dt, *J* 8.4, 4.1 Hz), –0.32 (1H, q, *J* 5.2 Hz);  $\delta_C$  (101 MHz, CDCl<sub>3</sub>): 175.0, 137.5, 137.3, 128.6, 128.5, 127.9, 127.8, 107.2, 87.9, 85.4, 83.7, 79.4, 72.4, 72.2, 72.1, 63.5, 57.7, 54.9, 51.5, 35.5, 35.3, 32.5, 32.3, 31.9, 31.3, 31.0, 30.9, 30.8, 30.79, 30.6, 30.58, 30.5, 30.47, 30.4, 30.38, 30.37, 30.3, 30.29, 30.2, 30.19, 30.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.18, 29.17, 29.16, 29.15, 29.0, 28.9, 28.8, 28.7, 28.5, 28.4, 28.3, 27.6, 27.4, 27.3, 26.9, 26.1, 25.7, 22.6, 15.7, 14.8, 14.1, 10.9;  $\nu_{max}$ : 3479, 3064, 2923, 2853, 1733, 1465, 1100, 721 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.003 g, 0.15 fold by weight) was added to a stirred solution of **7e** (0.020 g, 0.012 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then filtered and the solvent was evaporated to give a residue; column chromatography eluting with hexane/ethyl acetate (1:1) gave the title compound as a colorless oil **8e** (0.011 g, 65%) [Found (M+Na)<sup>+</sup>: 1394.3138, C<sub>89</sub>H<sub>174</sub>NaO<sub>8</sub> requires: 1394.3101], [ $\alpha$ ]<sub>D</sub><sup>16</sup> +10 (c 0.70, CHCl<sub>3</sub>);  $\delta_H$  (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.50 (1H, dd, *J* 12, 3.8 Hz), 4.32 (1H, dd, *J* 12, 4.4 Hz), 4.20 – 4.16 (1H, m), 4.07 (1H, d, *J* 5.6 Hz), 4.00 – 3.96 (1H, m), 3.74 – 3.64 (1H, m), 3.41 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.79 – 2.7 (2H, m), 2.48 – 2.39 (1H, m), 2.39 – 2.32 (1H, m), 1.6 – 1.05 (143H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H,

d,  $J$  6.9 Hz), 0.69 – 0.63 (2H, m), 0.60 – 0.53 (1H, dt  $J$ ), -0.32 (1H, br. q  $J$  4.5 Hz);  $\delta_{\text{C}}$  (101 MHz,  $\text{CDCl}_3$ ): 174.9, 108.7, 85.4, 83.8, 80.4, 78.4, 72.8, 63.2, 57.7, 55.0, 52.2, 35.3, 35.2, 32.3, 31.9, 30.5, 30.2, 30.0, 29.9, 29.7, 29.68, 29.6, 29.5, 29.4, 29.3, 28.7, 27.5, 27.4, 26.1, 25.4, 22.7, 15.7, 14.99, 14.1, 10.9;  $\nu_{\text{max}}$ : br. 3436, 2918, 2850, 1732, 1467, 1099, 720  $\text{cm}^{-1}$ .

**3.10: Methyl 5-*O*-(2-[(*R*)-1-hydroxy-18-[(1*R*, 2*S*)-2-[(17*S*, 18*S*)-17-methoxy-18-methylhexatriacetyl]cyclopropyl]octadecyl]hexacosanoate)  $\alpha$ -D-Araf **8f****

(a) Cesium hydrogen carbonate (0.083 g, 0.428 mmol) was added with stirring to **5** (0.0457 g, 0.091 mmol) and (*R*)-2-[(*R*)-1-hydroxy-18-[(1*R*, 2*S*)-2-[(17*S*, 18*S*)-17-methoxy-18-methylhexatriacetyl]cyclopropyl]octadecyl]hexacosanoic acid (0.076 g, 0.060 mmol)<sup>40</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and the mixture was stirred at 70 °C for two days then worked up as before to give methyl 2,3-*di-O*-benzyl-5-*O*-(2-[(*R*)-1-hydroxy-18-[(1*R*, 2*S*)-2-[(17*S*, 18*S*)-17-methoxy-18-methylhexatriacetyl]cyclopropyl]octadecyl]hexacosanoate) $\alpha$ -D-Araf **7f** (0.077 g, 80%) as a thick colorless oil [Found ( $\text{M}+\text{Na}$ )<sup>+</sup>: 1602.4304,  $\text{C}_{105}\text{H}_{190}\text{NaO}_8$  requires: 1602.4353],  $[\alpha]_{\text{D}}^{22}$  +18 (c 0.10,  $\text{CHCl}_3$ ), which showed  $\delta_{\text{H}}$  (400 MHz,  $\text{CDCl}_3$ ): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d,  $J$  12 Hz), 4.56 (1H, d,  $J$  12 Hz), 4.51 (1H, d,  $J$  12 Hz), 4.48 (1H, d,  $J$  12 Hz), 4.31 – 4.28 (2H, m), 4.26 – 4.19 (1H, m), 4.0 (1H, dd,  $J$  5.6, 2.5 Hz), 3.84 (1H, dd,  $J$  6.3, 2.5 Hz), 3.68 – 3.58 (1H, m), 3.37 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.50 (1H, d,  $J$  8.2 Hz), 2.44 (1H, dt,  $J$  8.7, 5.4 Hz), 1.75 – 1.56 (2H, m), 1.58 – 1.04 (145H, m), 0.89 (6H, t,  $J$  6.8 Hz), 0.86 (3H, d,  $J$  6.9 Hz), 0.70 – 0.62 (2H, m), 0.57 (1H, dt,  $J$  8.4 Hz), -0.32 (1H, br. q  $J$  5.0 Hz);  $\delta_{\text{C}}$  (101 MHz,  $\text{CDCl}_3$ ): 175.0, 137.4, 137.3, 128.5, 128.4, 128.0, 127.9, 127.89, 127.88, 127.87, 127.86, 107.2, 87.8, 85.4, 83.7, 79.4, 72.4, 72.2, 72.1, 63.4, 57.7, 54.9, 51.5, 35.5, 35.3, 32.4, 31.9, 30.8, 30.7, 30.67, 30.6, 30.55, 30.5, 30.45, 30.4, 30.38, 30.37, 30.36, 30.2, 30.17, 30.16, 30.15, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.27, 29.26, 29.2, 29.14, 29.1, 29.07, 29.02, 29.01, 28.94, 28.9, 28.7, 28.65, 28.6, 28.52, 28.5, 27.5, 27.4, 26.1, 25.7, 22.6, 15.7, 14.8, 14.1, 10.9;  $\nu_{\text{max}}$ : br. 3522, 3064, 2921, 2851, 1723, 1467, 1027, 732  $\text{cm}^{-1}$ .

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.003 g, 0.15 fold by weight) was added to a stirred solution of **7f** (0.020 g, 0.012 mmol) in dry  $\text{CH}_2\text{Cl}_2$  : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then worked up as before to give the title compound as a thick colorless oil **8f** (0.014 g, 76%) [Found ( $\text{M}+\text{Na}$ )<sup>+</sup>: 1422.3425,  $\text{C}_{91}\text{H}_{178}\text{NaO}_8$  requires: 1422.3414];  $[\alpha]_{\text{D}}^{21}$  +10 (c 0.10,  $\text{CHCl}_3$ );  $\delta_{\text{H}}$  (400 MHz,  $\text{CDCl}_3$ ): 4.89 (1H, s), 4.51 (1H, dd,  $J$  12, 3.9 Hz), 4.33 (1H, dd,  $J$  12.0, 4.1 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br. s), 4.01 – 3.95 (1H, m), 3.75 (1H, d,  $J$  5.3 Hz), 3.73 – 3.66 (1H, m), 3.41 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.85 – 2.70 (2H, m), 2.50 – 2.39 (1H, m), 1.72 – 1.61 (2H, m), 1.60 – 1.03 (145H, m), 0.89 (6H, t,  $J$  6.8 Hz), 0.85 (3H, d,  $J$  6.8 Hz), 0.70 – 0.61 (2H, m), 0.60 – 0.53 (1H, dt,  $J$  8.4 Hz), -0.33 (1H, br. q,  $J$  5.2 Hz);  $\delta_{\text{C}}$  (101 MHz,  $\text{CDCl}_3$ ): 175.0, 108.7, 85.5, 83.8, 80.4, 78.4, 72.8, 63.2, 57.7, 55.0, 52.2, 35.3, 35.2, 32.3, 31.9, 30.5, 30.2, 29.9, 29.89, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 28.7, 27.6, 27.4, 26.1, 22.4, 22.7, 15.77, 14.8, 14.1, 10.9;  $\nu_{\text{max}}$ : br. 3436, 2921, 2852, 1732, 1493, 1455, 759  $\text{cm}^{-1}$ .

**3.11: Methyl 5-*O*-(2-[(1*R*)-1-hydroxy-16-[(1*R*, 2*S*)-2-[20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]tetracosanoate)  $\alpha$ -D-Araf **8g****

(a) Cesium hydrogen carbonate (0.086 g, 0.443 mmol) was added to a stirred solution of **5** (0.0475 g, 0.0953 mmol) and (*R*)-2-[(*R*)-1-hydroxy-16-[(1*R*, 2*S*)-2-[(20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]-tetracosanoic acid (0.077 g, 0.063 mmol)<sup>41</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and stirred at 70 °C for two days. Work up as before gave methyl 2,3-*di-O*-benzyl-5-*O*-(2-[(1*R*)-1-hydroxy-16-[(1*R*, 2*S*)-2-[20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]tetracosanoate)  $\alpha$ -D-Araf as a thick colorless oil **7g** (0.077 g, 80%) [Found ( $\text{M}+\text{NH}_4$ )<sup>+</sup>: 1558.3708,  $\text{C}_{104}\text{H}_{190}\text{NO}_8$  requires: 1558.3727],  $[\alpha]_{\text{D}}^{21}$  +21 (c 0.10,  $\text{CHCl}_3$ );  $\delta_{\text{H}}$  (400 MHz,  $\text{CDCl}_3$ ): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d,  $J$  12.0 Hz), 4.56 (1H, d,  $J$  12 Hz), 4.51 (1H, d,  $J$  12 Hz), 4.48 (1H, d,  $J$  12 Hz), 4.32 – 4.28 (2H, m), 4.25 – 4.19 (1H, m), 3.99 (1H, br d,  $J$  2.2 Hz), 3.84 (1H, dd,  $J$  6.4, 2.8 Hz), 3.67 – 3.59 (1H, m), 3.38 (3H, s), 2.57 – 2.47 (2H, m), 2.47 – 2.38 (3H, m), 1.75 – 1.09 (140H, m), 1.06 (3H, d,  $J$  6.9 Hz), 0.89 (6H, t,  $J$  6.7 Hz), 0.70 – 0.62 (2H, m), 0.61 – 0.53 (1H, dt,  $J$  3.9, 7.9 Hz), -0.32 (1H, br. q,  $J$  5.2 Hz);  $\delta_{\text{C}}$  (101 MHz,  $\text{CDCl}_3$ ): 215.2, 175.0, 137.5, 137.3, 128.5, 128.4, 127.9, 127.9, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.1, 63.4, 54.9, 51.5, 46.3, 41.1, 33.0, 31.9, 30.2, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 28.7, 27.4, 27.3, 25.7, 23.7, 22.7, 16.3, 15.7, 14.1, 10.9;  $\nu_{\text{max}}$ : br. 3524, 3030, 3063, 2922, 2851, 1732, 1715, 1465, 1107, 733  $\text{cm}^{-1}$ .

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0015 g, 0.15 fold by weight) was added to a stirred solution of **7g** (0.010 g, 0.006 mmol) in dry  $\text{CH}_2\text{Cl}_2$  : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The reaction mixture was stirred overnight then worked up as before to afford the title compound as a thick white oil **8g** (0.0069 g, 63%) [Found ( $\text{M}+\text{NH}_4$ )<sup>+</sup>: 1401.3546,  $\text{C}_{90}\text{H}_{178}\text{NO}_8$  requires: 1401.3547],  $[\alpha]_{\text{D}}^{21}$  +10.5 (c 0.1,  $\text{CHCl}_3$  + few drops of  $\text{CD}_3\text{OD}$ );  $\delta_{\text{H}}$  (400 MHz,  $\text{CDCl}_3$ ): 4.89 (1H, s), 4.51 (1H, dd,  $J$  12, 4.0 Hz), 4.33 (1H, dd,  $J$  12.0, 4.1 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br. s), 4.02 – 3.95 (1H, m), 3.74 – 3.66 (1H, m), 3.41 (3H, s), 2.90 – 2.71 (2H, m), 2.56 – 2.48 (1H, m), 2.48 – 2.37 (4H, m, including the OH), 2.23 – 1.10 (140H, m), 1.05 (3H, d,  $J$  6.9 Hz), 0.89 (6H, t,  $J$  6.8 Hz), 0.70 – 0.61 (2H, m), 0.59 – 0.53 (1H, dt,  $J$  7.9, 3.9 Hz), -0.33 (1H, br. q,  $J$  5.2 Hz);  $\delta_{\text{C}}$  (101 MHz,  $\text{CDCl}_3$  + few drops of  $\text{CD}_3\text{OD}$ ): 215.0, 174.9, 108.7, 83.8, 80.4, 78.4, 72.8, 63.2, 54.9, 52.1, 46.3, 41.1, 35.2, 33.0, 31.9, 30.2, 29.7, 29.6, 29.5, 29.45, 29.4, 29.35, 29.3, 28.7, 27.4, 27.3, 25.4, 23.7, 22.7, 16.36, 15.7, 14.1, 10.9;  $\nu_{\text{max}}$ : br. 3436, 2918, 2850, 1731, 1708, 1467, 1170  $\text{cm}^{-1}$ .

**3.12: Methyl 5-*O*-(2-[(1*R*)-1-hydroxy-16-[(1*R*, 2*S*)-2-(20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]hexacosanoate)- $\alpha$ -D-Araf **8g****

(a) Cesium hydrogencarbonate (0.062g, 0.319 mmol) was added to a stirred solution of **5** (0.048 g, 0.096 mmol) and (*R*)-2-[(*R*)-1-hydroxy-16-[(1*R*, 2*S*)-2-[(20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]hexacosanoic acid (0.080 g, 0.064 mmol)<sup>42</sup> in dry DMF : THF (1:5, 4 mL) at room temperature and the mixture was stirred at 70 °C for 2 days, then worked up and purified as before to afford methyl 2,3-*di-O*-benzyl-5-*O*-(2-[(1*R*)-1-hydroxy-16-[(1*R*, 2*S*)-2-(20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]hexacosanoate)- $\alpha$ -D-Araf as a colourless thick oil **7g** (72 mg, 71%) [Found ( $\text{M}+\text{NH}_4$ )<sup>+</sup>: 1581.4467,  $\text{C}_{104}\text{H}_{190}\text{NO}_8$  requires: 1581.4486],  $[\alpha]_{\text{D}}^{23}$  + 25 (c 0.10,  $\text{CHCl}_3$ ) which showed  $\delta_{\text{H}}$  (400 MHz,  $\text{CDCl}_3$ ): 7.42 – 7.27 (10H, m), 4.92 (1H, s), 4.58 (1H, d,  $J$  12.0 Hz), 4.56 (1H, d,  $J$  11.8 Hz), 4.50 (1H, d,  $J$  12.0 Hz), 4.47 (1H, d,  $J$  11.8 Hz), 4.34 – 4.26 (2H, m),

4.22 (1H, dt,  $J$  4.6, 9.1 Hz), 3.99 (1H, br. d,  $J$  2.1 Hz), 3.84 (1H, dd,  $J$  2.6, 6.4 Hz), 3.68 – 3.58 (1H, m), 3.38 (3H, s), 2.56 – 2.47 (2H, m, including OH), 2.47 – 2.37 (3H, m), 1.76 – 1.09 (144H, m), 1.05 (3H, d,  $J$  6.9 Hz), 0.89 (6H, t,  $J$  6.8 Hz), 0.70 – 0.61 (2H, m), 0.56 (1H, dt,  $J$  4.0, 8.0 Hz), -0.33 (1H, br. q,  $J$  5.2 Hz);  $\delta_c$  (101 MHz,  $CDCl_3$ ): 215.2, 175.0, 137.4, 137.2, 128.5, 128.4, 128.0, 127.9, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.1, 72.0, 63.4, 54.9, 51.5, 46.3, 41.1, 35.5, 33.0, 31.9, 30.2, 29.7, 29.67, 29.6, 29.57, 29.5, 29.46, 29.4, 29.3, 28.7, 27.4, 27.3, 25.8, 23.7, 22.7, 16.4, 15.8, 14.1, 10.9;  $\nu_{max}$ : 3457, 3064, 3032, 2921, 2851, 1717, 1678, 1466, 1101, 755, 697  $cm^{-1}$ .

(b) Palladium hydroxide on activated charcoal (20%  $Pd(OH)_2 \cdot C$ , 0.01g, 0.15 fold by weight) was added to a stirred solution of compound **7g** (0.066 g, 0.042 mmol) in dry  $CH_2Cl_2$  : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was worked up and purified as before to afford the title compound **8g** as a colourless thick oil (39 mg, 66%) [Found (M+Na) $^+$ : 1401.3546,  $C_{90}H_{178}NO_8$ , requires: 1401.3547],  $[\alpha]_D^{25} +11$  (c 0.10,  $CHCl_3$ ) which showed  $\delta_H$  (400 MHz,  $CDCl_3$  + few drops  $CD_3OD$ ): 4.89 (1H, s), 4.51 (1H, dd,  $J$  3.9, 11.9 Hz), 4.32 (1H, dd,  $J$  4.0, 11.9 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br. s), 3.98 (1H, br. d,  $J$  1.8 Hz), 3.74 – 3.66 (1H, m), 3.41 (3H, s), 2.87 – 2.67 (2H, m), 2.55 – 2.38 (4H, m), 1.71 – 1.12 (145H, m), 1.05 (3H, d,  $J$  6.9 Hz), 0.89 (6H, t,  $J$  6.8 Hz), 0.69 – 0.61 (2H, m), 0.56 (1H, dt,  $J$  4.0, 8.0 Hz), -0.33 (1H, br. q,  $J$  5.2 Hz);  $\delta_c$  (101 MHz,  $CDCl_3$  + few drops  $CD_3OD$ ): 215.4, 175.0, 108.7, 83.6, 80.6, 78.4, 72.8, 63.2, 55.0, 52.3, 50.9, 46.3, 41.1, 35.2, 33.0, 31.9, 30.2, 29.7, 29.65, 29.6, 29.5, 29.45, 29.41, 29.40, 29.3, 28.7, 27.4, 27.3, 25.4, 23.7, 22.7, 16.4, 15.8, 14.1, 10.9;  $\nu_{max}$ : 3405, 2920, 2851, 1713, 1712, 1466, 1108, 759  $cm^{-1}$ .

### 3.13: Methyl 5-*O*-(2-((1*R*)-1-hydroxy-17-[(1*S*, 2*R*)-2-[(2*S*)-22-methyl-21-oxotetracontan-2-yl]cyclopropyl]heptadecyl]hexacosanoate) $\alpha$ -D-Araf **8h**

(b) Cesium hydrogen carbonate (0.045 g, 0.232 mmol) was added with stirring to **5** (0.025 g, 0.050 mmol) and (*R*)-2-((*R*)-17-[(1*S*, 2*R*)-2-[(1*S*)-1,21-dimethyl-20-oxo-nonatriacontyl]cyclopropyl]-1-hydroxy heptadecyl]hexacosanoic acid methyl ester (0.043 g, 0.033 mmol)<sup>43</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and stirred at 70 °C for two days, then worked up as before to give methyl 2,3-di-*O*-benzyl-5-*O*-(2-((*R*)-1-hydroxy-17-[(1*S*, 2*R*)-2-[(2*S*)-22-methyl-21-oxotetracontan-2-yl]cyclopropyl]heptadecyl]hexacosanoate)  $\alpha$ -D-Araf **7h** as a thick colorless oil (0.050 g, 92%) [Found (M+Na) $^+$ : 1628.4539,  $C_{107}H_{192}NaO_8$  requires: 1628.4509],  $[\alpha]_D^{25} +16.8$  (c 0.1,  $CHCl_3$ );  $\delta_H$  (400 MHz,  $CDCl_3$ ): 7.40 – 7.27 (10H, m), 4.92 (1H, s), 4.58 (1H, d,  $J$  12.0 Hz), 4.56 (1H, d,  $J$  12 Hz), 4.53 (1H, d,  $J$  12 Hz), 4.48 (1H, d,  $J$  12 Hz), 4.33 – 4.28 (2H, m), 4.22 (1H, dt,  $J$  10.7, 4.6 Hz), 3.99 (1H, br. d,  $J$  1.9 Hz), 3.84 (1H, dd,  $J$  6.4, 2.6 Hz), 3.68 – 3.58 (1H, dt,  $J$  7.6, 5.2 Hz), 3.38 (3H, s), 2.58 – 2.47 (2H, m), 2.47 – 2.38 (3H, m), 1.72 – 1.13 (146H, m), 1.06 (3H, d,  $J$  6.9 Hz), 0.89 (9H, including a triplet  $J$  7.5 Hz), 0.72 – 0.61 (1H, m), 0.50 – 0.41 (1H, m), 0.24 – 0.08 (3H, m);  $\delta_c$  (101 MHz,  $CDCl_3$ ): 215.2, 175.0, 137.5, 137.3, 128.5, 128.4, 128.0, 127.9, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.1, 63.5, 54.9, 51.5, 46.3, 41.1, 38.1, 37.4, 35.5, 34.5, 33.0, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 27.4, 27.35, 27.3, 26.1, 25.7, 23.7, 22.7, 19.7, 18.6, 16.3, 14.1, 10.5;  $\nu_{max}$ : br. 3524, 3064, 3031, 2923, 2853, 1737, 1715, 1465, 1107, 734  $cm^{-1}$ .

(b) Palladium hydroxide on activated charcoal (20%  $Pd(OH)_2 \cdot C$ , 0.0015 g, 0.15 fold by weight) was added to a stirred solution of **7h** (0.010 g, 0.006 mmol) in dry  $CH_2Cl_2$  : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then worked up as before to afford the title compound as a thick colorless oil **8h** (0.010 g, 80%) [Found

(M+Na) $^+$ : 1448.3585,  $C_{93}H_{180}NaO_8$  requires: 1448.3570],  $[\alpha]_D^{25} +13$  (c 0.10,  $CHCl_3$ );  $\delta_H$  (400 MHz,  $CDCl_3$ ): 4.89 (1H, s), 4.49 (1H, dd,  $J$  12, 4 Hz), 4.33 (1H, dd,  $J$  12, 4.2 Hz), 4.21 – 4.16 (1H, m), 4.09 (1H, d,  $J$  6.8 Hz), 4.01 – 3.95 (1H, m), 3.75 (1H, d,  $J$  5.4 Hz), 3.73 – 3.65 (1H, m), 3.41 (3H, s), 2.61 – 2.47 (1H, m), 2.46 – 2.28 (5H, m), 1.72 – 1.15 (146H, m), 1.06 (3H, d,  $J$  6.9 Hz), 0.89 (9H, including a triplet at 0.89 with  $J$  7.4 Hz), 0.76 – 0.61 (1H, m), 0.51 – 0.39 (1H, m), 0.26 – 0.05 (3H, m);  $\delta_c$  (101 MHz,  $CDCl_3$ ): 215.3, 174.9, 108.7, 83.8, 80.4, 78.8, 78.4, 72.8, 54.9, 52.2, 46.3, 44.5, 41.1, 38.1, 37.4, 35.2, 34.5, 33.0, 31.9, 30.0, 29.7, 29.6, 29.5, 29.3, 27.3, 26.1, 25.4, 23.71, 22.7, 19.7, 18.6, 16.3, 14.1, 10.5;  $\nu_{max}$ : br. 3467, 2917, 2849, 1731, 1713, 1467, 1050, 720  $cm^{-1}$ .

### 3.14: Methyl 5-*O*-(2-((*R*)-1-hydroxy-12-[(1*S*, 2*R*)-2-[(14-[(1*S*, 2*R*)-2-eicosylcyclopropyl]tetradecyl]cyclopropyl]dodecyl]-hexacosanoate) $\alpha$ -D-Araf **8i**

(a) Cesium hydrogen carbonate (0.089g, 0.46 mmol) was added to a stirred solution of **5** (0.049 g, 0.098 mmol) and (*R*)-2-((*R*)-1-hydroxy-12-[(1*S*, 2*R*)-2-[(14-[(1*S*, 2*R*)-2-eicosylcyclopropyl]tetradecyl]cyclopropyl]dodecyl]hexacosanoic acid (0.075 g, 0.065 mmol)<sup>37</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and then stirred at 70 °C for two days. Work up as before gave methyl 2,3-di-*O*-benzyl-5-*O*-(2-((*R*)-1-hydroxy-12-[(1*S*, 2*R*)-2-[(14-[(1*S*, 2*R*)-2-eicosylcyclopropyl]tetradecyl]cyclopropyl]dodecyl]hexacosanoate)  $\alpha$ -D-Araf **7i** as a thick colorless oil (0.084 g, 87%) [Found (M+Na) $^+$ : 1486.3173,  $C_{98}H_{174}NaO_7$  requires: 1486.3152],  $[\alpha]_D^{25} +31$  (c 0.10,  $CHCl_3$ );  $\delta_H$  (400 MHz,  $CDCl_3$ ): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d,  $J$  12 Hz), 4.56 (1H, d,  $J$  12 Hz), 4.51 (1H, d,  $J$  12 Hz), 4.48 (1H, d,  $J$  12 Hz), 4.32 – 4.28 (2H, m), 4.25 – 4.19 (1H, m), 3.99 (1H, br. d,  $J$  2.3 Hz), 3.84 (1H, dd,  $J$  6.4, 2.6 Hz), 3.68 – 3.59 (1H, m), 3.38 (3H, s), 2.52 (1H, d,  $J$  8.3 Hz), 2.44 (1H, dt,  $J$  9.3, 5.5 Hz), 1.62 – 1.03 (134H, m), 0.89 (6H, t,  $J$  6.7 Hz), 0.72 – 0.62 (4H, m), 0.61 – 0.52 (2H, dt,  $J$  8, 4 Hz), -0.32 (2H, br. q,  $J$  5.1 Hz);  $\delta_c$  (101 MHz,  $CDCl_3$ ): 175.0, 137.4, 137.2, 128.5, 128.4, 128.0, 127.9, 127.89, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.2, 72.1, 63.5, 54.9, 51.5, 35.5, 31.9, 30.55, 30.5, 30.45, 30.44, 30.4, 30.35, 30.3, 30.28, 30.2, 30.18, 30.1, 30.0, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.29, 29.2, 29.1, 29.03, 29.0, 28.7, 28.6, 27.4, 25.7, 22.68, 22.6, 15.7, 14.1, 10.9;  $\nu_{max}$ : 3479, 3065, 2989, 2919, 2849, 1733, 1607, 1494, 718  $cm^{-1}$ .

(b) Palladium hydroxide on activated charcoal (20%  $Pd(OH)_2 \cdot C$ , 0.0087 g, 0.15 fold by weight) was added to a stirred solution of **7i** (0.058g, 0.039 mmol) in dry  $CH_2Cl_2$  : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then worked up as before to afford the title compound as a thick colorless oil **8i** (0.040 g, 80%) [Found (M+Na) $^+$ : 1306.2201,  $C_{84}H_{162}NaO_7$  requires: 1306.2213],  $[\alpha]_D^{25} +12$  (c 0.10,  $CHCl_3$ );  $\delta_H$  (400 MHz,  $CDCl_3$  + few drops of  $CD_3OD$ ): 4.89 (1H, s), 4.47 (1H, dd,  $J$  11.8, 4.2 Hz), 4.37 (1H, dd,  $J$  11.9, 4.1 Hz), 4.20 – 4.14 (1H, m), 4.07 (1H, br. s), 3.98 (1H, br. s), 3.79 – 3.67 (1H, m), 3.40 (3H, s), 3.06 (2H, br. s), 2.45 (1H, dt,  $J$  10.1, 5.0 Hz), 1.90 – 1.83 (1H, m), 1.63 – 1.07 (134H, m), 0.89 (6H, t,  $J$  6.8 Hz), 0.71 – 0.61 (4H, m), 0.60 – 0.49 (2H, dt,  $J$  8, 4 Hz), -0.33 (2H, br. q,  $J$  5.2 Hz);  $\delta_c$  (101 MHz,  $CDCl_3$ ): 175.0, 108.7, 83.4, 80.7, 78.4, 72.8, 63.2, 54.9, 52.4, 35.1, 31.9, 30.4, 30.2, 30.18, 29.7, 29.68, 29.6, 29.5, 29.48, 29.4, 29.35, 29.3, 28.7, 27.4, 25.4, 22.6, 15.7, 14.1, 10.9;  $\nu_{max}$ : 3436, 390, 2918, 2850, 1733, 1467, 1455, 1050  $cm^{-1}$ .

### 3.15: The ELISA assay



ELISA were carried out on 96-well flat-bottomed polystyrene micro-plates. Antigens were dissolved in hexane to give an antigen solution of concentration 15 µg/ml. 50 µl of this solution was added to each well, and the solvent was left to evaporate at room temperature. Control wells were coated with hexane (50 µl / well) only. Blocking was done by adding 400 µl of 0.5 % casein/PBS buffer (pH 7.4) to each well, and the plates were incubated at 25 °C for 30 min. The buffer was aspirated and any excess buffer was flicked out until the plates were dry. Serum (1 in 80 dilution in casein/PBS buffer) (50 µl / well) was added and incubated at 25 °C for 1 h. The plates were washed with 400 µl casein/PBS buffer 3 times using an automatic washer, and any excess buffer was flicked out onto a paper towel until dry. Secondary antibody (anti-human IgG (Fc specific) peroxidase conjugated antibody produced in goat (Aldrich) (diluted to a concentration of 1:2000 in casein/PBS buffer) (50 µl / well) was added, and incubated at 25 °C for 30 minutes. The plates were again washed 3 times with 400 µl casein/PBS buffer using an automatic washer, and any excess buffer was again flicked out. OPD substrate (50 µl / well) (*o*-phenylenediamine (1 mg / ml) and H<sub>2</sub>O<sub>2</sub> (0.8 mg / ml) in 0.1 M citrate buffer) was then added, and the plates were incubated for a further 30 minutes at 25 °C. The colour reaction was terminated by adding 2.5 M H<sub>2</sub>SO<sub>4</sub> (50 µl / well), and the absorbance was read at 492nm.

## Acknowledgments

We thank the World Health Organisation, through Dr A. Ramsay, for making available a set of serum samples with associated information as to their TB status. MM wishes to thank the Ministry of Higher Education & Scientific Research - Kurdistan Region, Iraq for the award of a grant.

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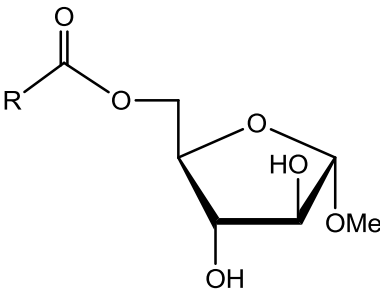


# Graphical Abstract

## Arabino Mycolates from Synthetic Mycolic Acids

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R = synthetic mycolic acid



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## Arabino Mycolates from Synthetic Mycolic Acids

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### ARTICLE INFO

### ABSTRACT

#### Article history:

Received

Received in revised form

Accepted

Available online

The synthesis of single mono-arabino mycolates, important lipid antigens from mycobacteria is described, using structurally defined synthetic mycolic acids. Preliminary assays indicate that these are differentially antigenic to antibodies in the serum of people with active tuberculosis.

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#### Keywords:

Mycobacteria

Tuberculosis

Arabino mycolates

Antigens

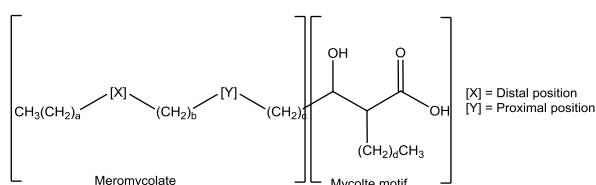
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## 1. Introduction

The mycobacterial cell wall has a complex structure made up of lipids, glycolipids, polysaccharides and proteins.<sup>1</sup> There are four major components: peptidoglycan (PG), mycolyl-arabinogalactan (mAG), lipoarabinomannan (LAM) and extractable lipids.<sup>2</sup> The mycolyl-arabinogalactan (mAG) complex is the largest component structure and acts as a permeability barrier that prevents passage of antibiotics. It forms from cross bonding between both D-arabinofuranosyl (Araf) and-galactofuranosyl (Galf) with a long chain (C70-C90),  $\alpha$ -alkyl branched  $\beta$ -hydroxylated fatty acid, 'mycolic acid'; carbohydrate (Araf) and (Galf) are bound to peptidoglycan in the cell wall by an  $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy- $\alpha$ -D-glucopyranosyl phosphate disaccharide. The galactan part is linear and composed of alternating  $\beta$ -(1 $\rightarrow$ 5) and  $\beta$ -(1 $\rightarrow$ 6) galactofuran residues. Galactan and arabinan are bonded from C-5 of the  $\beta$ -(1 $\rightarrow$ 6) link.<sup>3-6</sup> Both galactosyl and arabinosyl units in mAG are in a furanose form, less stable thermodynamically than the pyranose form.<sup>7</sup> It is believed that this plays a large role in raising the flexibility of the polysaccharide and making the mycolic acids (MAs) pack strongly by van de Waals interaction. Thereby, the structure of the cell wall has extremely low permeability and this provides the organism with high protection from drugs and from its environment.<sup>1</sup> Therefore, studying and understanding mAG biosynthesis is an important strategy for developing new anti-tuberculosis drugs. Indeed, isoniazid and ethambutol, two of the standard antibiotics, target mAG biosynthesis; ethambutol inhibits arabinosyltransferases which contribute to the biosynthesis of the arabinan part of the polysaccharides while isoniazid inhibits mycolic acid biosynthesis.<sup>8</sup>

It is approaching a century since Anderson began his ground breaking studies of the lipid components of mycobacteria, leading eventually to the characterization of MAs. These were shown to be high-molecular weight hydroxy acids, with an experimental formula of  $C_{88}H_{172}O_4$  or  $C_{88}H_{176}O_4$  which were very hard to purify and not possible to crystallize.<sup>9,10</sup> The long alkyl branch on the  $\alpha$ -position and hydroxyl groups in  $\beta$ -position in MAs was proved by Asselineau in 1950.<sup>11</sup> In brief, MAs can be defined as a complex group of long chain fatty  $\alpha$ -alkyl  $\beta$ -alkyl hydroxyl fatty acids (**Figure 1**).<sup>12,13</sup>



**Figure 1:** Generalised mycolic acid structure

The proximal group Y is generally a *cis*-alkene or cyclopropane, or a *trans*-alkene or cyclopropane with a further methyl substituent on the adjacent carbon distal from the hydroxyl acid. The distal group X is normally a *cis*-cyclopropane ( $\alpha$ -MAs), a  $-(CHMeCHOMe)-$  (methoxy MAs) or a  $-(CHMeCO)-$  fragment (keto MAs). Mixtures extracted from mycobacteria comprise many individual MA containing a range of functional groups X and Y (Figure 1) and but each also of several chain lengths.<sup>14</sup>

Anderson & Geiger in 1937 claimed the first extraction of arabino-mycolate from the cell wall of *Mycobacterium bovis* using natural organic solvent.<sup>15</sup> Some fifty years ago, the isolation of arabinose 5-mycolate by extraction of the cell walls

of various mycobacteria under acidic conditions was reported.<sup>16-22</sup> More recently, mass spectrometry and NMR have provided powerful tools for the analysis of such molecules.<sup>23</sup> Azuma and Yamamura in 1962 isolated arabinomycolate as a D-arabinose-5-mycolate and proved it was toxic to mice.<sup>17</sup> Inflammatory reactions similar to that observed after inoculation of live BCG were induced in the lungs by trehalose monomycolate and dimycolate (TMM and TDM) or glucose monomycolate (GMM) isolated from BCG. However, the toxic reactions caused by glycerol monomycolate (GroMM) and arabinose mono-mycolate were characterized by an acute inflammatory process.<sup>24</sup>

In 2006, the preparation of a tetramycolyl pentaarabinose using a complex natural mixture of MAs was described.<sup>25</sup> However, only in 2010 were structural studies of the composition of the arabinose mycolates of the cell wall of *M. bovis* reported. A two-layer acid hydrolysis gave a number of fractions. One of these was a penta-arabinose tetramycolate, one was an arabinose mono-mycolate, while the others were hexa-arabinose, hepta-arabinose and octa-arabinose tetramycolates. The mycolic acid methyl esters released from each of these showed a mass spectrometric pattern almost identical to the methyl esters obtained by hydrolysis of the original cell wall. A comparison of the penta-arabinose tetramycolate and arabinose monomycolate with samples prepared by combining a mixture of natural MAs with arabinose was reported.<sup>26</sup> Ishiwata *et al* reported the synthesis of a series of mono-, di- and tetra-arabinomycolates found in the terminal position of cell wall skeleton of bacillus Calmette Guerin from *M. bovis*, by using natural mycolic acid mixtures extracted from the cell wall. They proved the biological activity for synthesized compounds in a tumor necrosis factor alpha (TNF- $\alpha$ ) secretion-inducing assay; all the compounds showed strong TNF- $\alpha$  inducing activity *in vitro*.<sup>25</sup> The mechanism of the activity of arabino-mycolate is not clear.<sup>22</sup> Synthetic arabino-mycolates induce the production of TNF- $\alpha$  in murine macrophage cell lines at an intensity similar to BCG-CWS (cell wall skeleton). However the immunological activity of natural arabino-mycolates isolated from BCG has not been investigated, probably due to the complexity of the molecule. Arabino-mycolates obtained by acid hydrolysis from CWS (SMP-105) of *M. bovis* BCG Tokyo 172 strain consisted mainly of mono-arabinose mono-mycolate, pentaarabinose tetramycolate and hexa-arabinose tetramycolate fractions. Arabino-mycolates significantly induced TNF- $\alpha$  production and enhanced delayed type hypersensitivity (DTH) reactions against inactivated tumour cells. Arabino-mycolates-induced TNF- $\alpha$  production was completely dependent on TLR2 and MyD88 pathways. Thus isolated natural arabino-mycolates possess potent adjuvant immunostimulatory activity.<sup>27</sup>

Intra-tumor injections of extracts of Re mutant *Salmonella typhimurium* in combination with TDM or arabinose mycolate were highly effective in producing regression of tumors in guinea pigs. Similar extracts from *M. bovis* strain BCG and strain AN5 in combination with TDM also possessed tumor-regressive activity. The activity was reduced when the arabinose mycolate was substituted for the TDM. An extract of *Coxiella burnetii*, in combination with either TDM or arabinose mycolate was also active. Intracutaneous administration of Re glycolipid or aqueous extracts from BCG in combination with trehalose or arabinose mycolates did not produce life-threatening, clinical signs of toxicity in young mice. If additional toxicity studies demonstrate that adverse side effects can be satisfactorily controlled, these water soluble extracts may prove beneficial in the treatment of spontaneous tumors of humans and other animals.<sup>28,29</sup> The adjuvant activity of cell wall skeletons (mycolic acid-arabino-galactan-mucopeptide) prepared from the cells of mycobacteria,

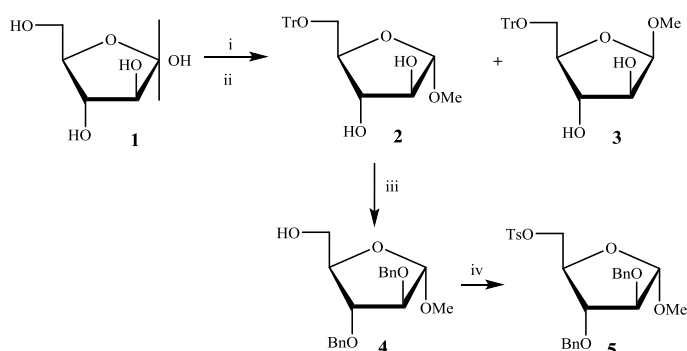
nocardia and corynebacteria was examined in vivo in mice and guinea pigs. The cell wall skeletons of *M. bovis* BCG (BCG-CWS), *Nocardia asteroides* 131 and *Corynebacterium diphtheriae* PWC suspended in Freund's incomplete adjuvant (FIA) as water-in-oil emulsions showed potent adjuvant activity on the formation of circulating antibody and cell-mediated immunity to bovine serum albumin (BSA), sheep erythrocytes (SRBC) and sulfanylazo-bovine serum albumin (SA-BSA) in mice and guinea pigs. After acetylation or acid treatment, BCG-CWS retained its adjuvant activity, but the activity of BCG-CWS was destroyed completely by alkaline treatment. The cell wall constituents, arabinose-mycolate and arabino-galactan, prepared from BCG-CWS showed no adjuvant activity.<sup>30</sup>

Few studies of arabinose mycolates have been carried out and little is known of the effect of structure on the immunostimulatory activity of arabino-mycolates in activating macrophages.<sup>6</sup> However, D-arabinose-5-mycolate, purified from bound lipids of the cell-wall skeleton of *M. bovis* BCG may be prominent structure for recognition by host immunity.<sup>6</sup>

However, it is now clearly established that, in the case of free MAs, the detailed structure controls their effects on a range of cytokines and chemokines and that some lead to inflammatory reactions on intratracheal instillation in mice, while others lead to the formation of foam cells.<sup>31</sup> We now report the synthesis of a set of arabinose esters of stereochemically defined synthetic MAs, as well as those of some simple fatty acids.

## 2a Results and discussion: synthesis of arabinose mycolates

According to literature procedures, D-(-)-arabinose **1** was treated with HCl (0.22 M) freshly prepared by addition of acetyl chloride to anhydrous methanol at 0 °C working up with pyridine rather than ammonium carbonate,<sup>32</sup> to give methyl- $\alpha,\beta$ -D-arabino furanoside with predominant formation of the  $\alpha$ -anomer ( $\alpha$ -D/ $\beta$ -D 3:2).<sup>33,34</sup> In order to isolate these two anomers, *in situ* tritylation of this mixture followed by column chromatography gave methyl 5-O-trityl  $\alpha$ -D-Araf **2** (45%) and 5-O-trityl  $\beta$ -D-Araf **3** (30%). Compound **2** was benzylated to protect the two secondary hydroxyl groups by using benzyl bromide and sodium hydride in dry DMF then the trityl group was removed from the primary hydroxyl group by hydrolysis in 80% AcOH, affording compound **4** (Scheme 1).<sup>25,34</sup>



**Scheme 1.** Reagents and conditions: (i) HCl, CH<sub>3</sub>OH; (ii) Trityl chloride, DMAP, pyridine, 0 °C/R.T., then 70 °C 4 h, 45% from  $\alpha$ -anomer; (iii) a: NaH, BnBr, DMF, 0 °C/R.T. 2 h, b: 80% AcOH, 75 °C for 4 h, 78%; (iv) 4-toluenesulfonyl chloride, DMAP, pyridine, 90%.

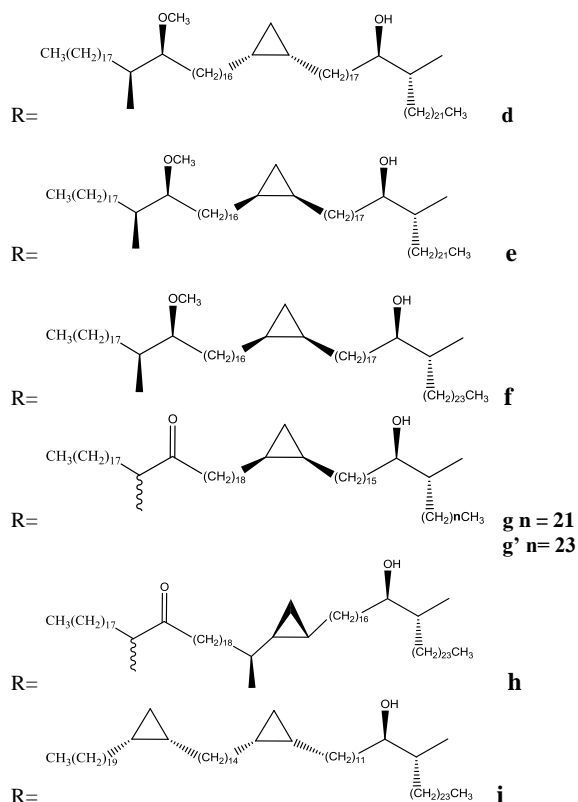
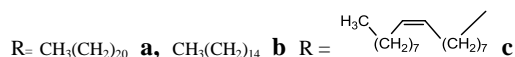
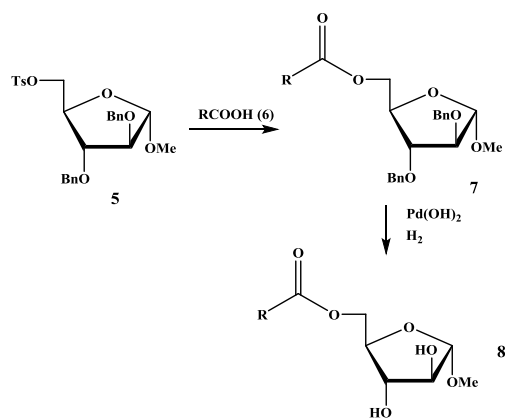
According to the literature,<sup>25</sup> direct esterification between the primary hydroxyl group of Araf and a carboxyl group in natural mycolic acid mixtures was achieved in a low yield (30%), while

activating the sugar as a tosylate raised the yield to 79%. Therefore, the hydroxyl group in the compound **4** was tosylated by reaction with p-toluenesulfonyl chloride in dry pyridine and catalytic 4-dimethylaminopyridine (DMAP) in dry CH<sub>2</sub>Cl<sub>2</sub> at 0 °C to afford the tosylate **5**. Surprisingly, esters of arabinose with simple fatty acids do not appear to have been reported. In a model experiment, compound **4** was therefore first condensed with behenic acid using DMAP and N,N-dicyclohexylcarbodiimide in dry CH<sub>2</sub>Cl<sub>2</sub> to give compound **7a** in 80% yield.<sup>36</sup> This was then hydrogenolysed in the presence of Pd(OH)<sub>2</sub> and under a hydrogen atmosphere to give the behenyl arabinose **8a**. Compound **5** was also reacted with palmitic acid by an alkylative esterification using cesium hydrogen carbonate in dry DMF: THF at 70 °C,<sup>25</sup> to give compound **7b**; this was then debenzylated to give the compound **8b**. In the same way, compound **5** was reacted with oleic acid to give the compound **7c**; hydrogenolysis in the presence of Pd(OH)<sub>2</sub> under a hydrogen atmosphere gave the corresponding stearyl arabinose, in which the double bond was also saturated. However, deprotection with boron trichloride in dichloromethane and methanol gave the oleoyl derivative **8c**.

A series of synthetic MAs was then reacted with the compound **5** by alkylative esterification using cesium hydrogen carbonate as in Scheme 2. Firstly, the methoxy *cis*-cyclopropane mycolic acid **6d**,<sup>37</sup> gave compound **7d**, which was then debenzylated using hydrogen and Pd(OH)<sub>2</sub> catalyst to give the arabinose ester **8d**. This was achieved with no loss of the cyclopropane group, on the basis of the proton and carbon NMR spectra. Although the absolute stereochemistry of the *cis*-cyclopropane unit in MAs remains unproven, this compound contains the stereochemistry that appears likely, based on a common intermediate leading to the different classes of mycolic acid.<sup>38</sup> The 22 carbon length of the  $\alpha$ -chain is typical of mycobacteria such as *Mycobacterium kansasii*.<sup>39</sup> Condensation of methoxy-*cis*-cyclopropane mycolic acid **6e**,<sup>37</sup> having a twenty two carbon chain in the  $\alpha$ -position, and the opposite absolute *cis*-cyclopropane stereochemistry, one stereoisomer of which is present in *M. kansasii*, led to compound **7e**. On hydrogenolysis as above, this gave compound **8e**. In the same way, esterification of methoxy-*cis*-cyclopropane mycolic acid **6f**,<sup>40</sup> with a twenty four carbon chain at the  $\alpha$ -position, a stereoisomer of which is a major component of *M. tuberculosis* methoxy-MA,<sup>39</sup> but the opposite cyclopropane stereochemistry to **6d** gave compound **7f**, which on hydrogenolysis led to compound **8f**.

The same procedure was repeated to prepare the arabino ester compounds for the keto *cis*-cyclopropane mycolic acid **6g**,<sup>37</sup> with a C<sub>22</sub> alkyl chain in the  $\alpha$ -position; this was used as the epimeric mixture at the carbon adjacent to the ketone. This produced compound **7g** which on debenzylation gave the arabinose ester **8g**. Ester **8g'**, with the normal C<sub>24</sub> *M. tuberculosis*  $\alpha$ -chain length, was also obtained from **6g'**.<sup>41</sup> In the same way the arabinose ester **8h**, from the keto *trans*-cyclopropane mycolic acid **6h**,<sup>42</sup> again epimeric adjacent to the ketone, was obtained in good yield. Finally, the  $\alpha$ -mycolic acid **6i**,<sup>43,44</sup> was esterified to give compound **7i** and then debenzylation to give **8i**.

The yields for each of these coupling steps and deprotections are given in Table 1. All the products were characterized by accurate mass MALDI-MS, proton and carbon NMR and the spectra are presented as Supplementary Information.



**Scheme 2:** Synthesis of arabinose esters

R	<b>7</b> (%)	<b>8</b> (%)
a	80	81
b	76	88
c	83	64
d	77	79
e	75	65
f	80	76
g	80	63
g'	71	66
h	92	80
i	87	80

**Table 1:** Yields for arabinose esters

## 2b. Assessment of arabinose mycolates as antigens

It is known that both trehalose esters of natural MAs, and the free acids themselves are antigenic to antibodies in the serum of patients infected with tuberculosis (TB).<sup>45,46</sup> As an initial study of the biological properties of the unique arabinose mycolates prepared in this work, their antigenicity to 64 serum samples taken from a Gambian population was determined using a standard enzyme-linked immunosorbent assay (ELISA). The samples, provided by the World Health Organisation from the TDR TB Specimen Bank,<sup>47</sup> were all from individuals with symptoms of suspected tuberculosis. Nine were diagnosed as having active TB using a range of clinical and biochemical assays; 55 as were diagnosed as not having active TB. The categorization was carried out using standard WHO protocols. The assay used an IgG(Fc) conjugated secondary antibody to detect the disease antibody – antigen interaction based on the resulting absorbance at 492 nm. The results are summarized in **Table 2**. This shows the median absorbance for active TB and no active TB samples, the area under the curve (AUC) for a Receiver Operating Characteristic (ROC) analysis of the data for each antigen, the cut-off response set for a positive response for each antigen in the assay, and the resulting sensitivity (% of active TB diagnosed samples giving a response above the assay cut-off) and specificity (% of no active TB samples giving a response below the cut-off value).

	7d	7e	7f	7g	7h	7i
Median active TB	0.64	0.40	0.78	0.50	0.48	1.18
Median no active TB	0.39	0.33	0.46	0.44	0.45	0.52
ROC AUC	.85	.69	.87	.69	.67	.95
Cut-off for 'active' in assay	>.35	>.27	>.45	>1.00	>.25	>.7
Active TB samples above the cut-off	9	9	9	1	9	9
No active TB samples above the cut-off	18	29	20	2	45	11
Sensitivity	100	100	100	100	100	100
Specificity	67	47	64	20	18	80

**Table 2.** Responses for a set of 64 human serum samples from Gambia (9 diagnosed with active TB, 55 with no active TB) to synthetic arabinose mycolates coated as antigens on ELISA plates, using IgG(Fc) secondary antibody conjugate to determine the binding. The closer the ROC AUC is to 1, the higher the predictive value of the assay.

This shows that the six synthetic arabinose mycolates are detected to a different degree by antibodies in the serum of patients infected with active tuberculosis; in some cases the response is essentially no different between active TB and no active TB samples, whereas in others, such as **7i**, the assay result correlates well with the clinical diagnosis. The active TB status had been determined using a range of standard assays employed by the WHO, including being smear and culture positive; the no active TB samples were all from patients showing some of the symptoms of TB, but diagnosed using the same methods as not having active TB, and being both smear and culture negative. Although this is a very small sample set, it is interesting that the highest distinction between active TB and no active TB sets is given with the arabinose mycolates **7d** and **7i** which probably represent the natural stereochemistries of methoxy- and  $\alpha$ -mycolates. Work is on-going to validate these results on a larger sample set.

The effects of selected examples of the above synthetic arabinose mono-mycolates on the Mincle receptor in *M. tuberculosis* will be described elsewhere.<sup>48</sup>

### 3. Experimental section

Chemicals used were obtained from commercial suppliers (Sigma, Aldrich, and Alfa Aesar) or prepared from them by the methods described. Solvents which were required to be dry, e.g. ether, tetrahydrofuran were dried over sodium wire and benzophenone under nitrogen, while dichloromethane and HMPA were dried over calcium hydride. All reagents and solvents used were of reagent grade unless otherwise stated. Organic solutions were dried over anhydrous magnesium sulfate.

Silica gel (Merck 7736) and silica gel plates used for column chromatography and thin layer chromatography were obtained from Aldrich; separated components were detected using variously UV light,  $I_2$  and phosphomolybdic acid solution in IMS followed by charring. Infra-red (IR) spectra were carried out on a Perkin-Elmer 1600 F.T.I.R. spectrometer as liquid films or KBr disc (solid). Melting points were measured using a Gallenkamp melting point apparatus. NMR spectra were carried out on a Bruker AC250 or Advance 400 spectrometer.  $[\alpha]_D$  values were recorded in  $CHCl_3$  on a POLAAR 2001 optical activity polarimeter. Low resolution mass spectra were recorded on a Bruker matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) values are given plus sodium. Accurate mass measurements were carried out by the EPSRC UK National Mass Spectrometry Facility at Swansea University.

#### 3.1: Methyl 5-*O*-trityl- $\alpha$ -D-Araf 2

Freshly prepared HCl solution in MeOH (resulting from mixing acetyl chloride (2 mL) in MeOH (30 mL) at 0 °C) was added to a stirred solution of D-(-)-arabinose (5.0 g, 33 mmol) in anhydrous MeOH (100 mL). Stirring was continued overnight at room temperature, when a clear solution was obtained. The mixture was neutralized by adding pyridine to pH 7–8, and the solid was filtered and washed with MeOH (10 mL). The solvent was evaporated to give a residue which was purified by column chromatography eluting with chloroform: acetone (3:5) to give a colourless oil, methyl- $\alpha,\beta$ -D-Araf (4.3 g, 78%). Trityl chloride (6.42 g, 23.0 mmol) and 4-(dimethylamino)pyridine (2.57 g, 21.0 mmol) were added to a stirred solution of methyl- $\alpha,\beta$ -D-Araf (3.12 g, 19.0 mmol) in anhydrous pyridine (60 mL) and the mixture was stirred at room temperature overnight then heated on oil bath at 70 °C for 4 h. The mixture was cooled to room temperature and poured into ice/water (300 mL). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3  $\times$  100 mL). The combined organic layers were washed with aq.  $NaHCO_3$  solution (5%, 100 mL), dried and the solvent was evaporated. The residue was purified by column chromatography eluting started from 10% to 50% hexane/ethyl acetate gave the title compound as a colorless oil **2** (3.3 g, 45%) and 5-*O*-trityl- $\beta$ -D-Araf **3** (2.3 g, 30%). The mixture of anomers showed  $\delta_H$  (400 MHz,  $CDCl_3$ ),  $\delta_C$  (125 MHz,  $CDCl_3$ ) and  $\nu_{max}$  identical to the literature.<sup>33,34</sup>

#### 3.2: Methyl 2,3-di-*O*-benzyl- $\alpha$ -D-Araf 4

A solution of **2** (3.20 g, 7.88 mmol) in dry DMF (80 mL) was added dropwise to a stirred suspension of NaH (0.85 g) (60% w/w, dispersion in mineral oil, washed with petrol three times) at room temperature under nitrogen. The mixture was stirred for 30 min. then benzyl bromide (2.5 mL, 3.6 g, 21.0 mmol) in dry DMF (50 mL) was added. The mixture was stirred at room temperature for 20 h and then quenched by slow addition of water (15 mL) and diluted with ether (25 mL). The organic layer was separated and the aqueous layer was extracted with ether (2  $\times$  100 mL). The combined extracts were washed with water (50 mL), brine (50 mL), dried and evaporated under reduced pressure. Aqueous acetic acid (80%, 30 mL) was added to the crude product and the mixture was stirred and heated at 75 °C for 4 h, then diluted with water (20 mL) and ether (20 mL) and the organic layer was separated and the aqueous layer extracted with ether (2  $\times$  100 mL). The combined organic layers were washed with water (50 mL), sat. aq.  $NaHCO_3$  (50 mL) and brine (50 mL), dried and the solvent was evaporated. Column chromatography eluting with petrol/ethyl acetate (7:3) gave the title compound as

a colorless oil **4** (2.12 g, 78%) [MALDI-Found (M+Na)<sup>+</sup>: 367.3; C<sub>20</sub>H<sub>24</sub>NaO<sub>5</sub>, requires: 367.1], [α]<sub>D</sub><sup>22</sup> +89 (c 0.10, CHCl<sub>3</sub>) [lit.<sup>26</sup> [α]<sub>D</sub><sup>24</sup> +83.2 (c 1.14, CHCl<sub>3</sub>)], which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.40 – 7.28 (10H, m), 4.95 (1H, s), 4.61 (1H, d, *J* 12.0 Hz), 4.54 (1H, d, *J* 12 Hz), 4.53 (1H, d, *J* 12 Hz), 4.50 (1H, d, *J* 12 Hz), 4.18 – 4.12 (1H, m), 4.02 – 3.96 (2H, m), 3.85 (1H, dd, *J* 12.1, 2.8 Hz), 3.65 (1H, dd, *J* 12.1, 4.1 Hz), 3.40 (3H, s), 2.93 (1H, br. s); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 137.6, 137.2, 128.4, 128.4, 127.9, 127.8, 127.7, 107.3, 87.6, 82.5, 82.2, 72.3, 71.8, 62.1, 54.8; ν<sub>max</sub>: 3466 br., 3089, 3064, 3031, 2925, 1725, 1605, 1454, 739 cm<sup>-1</sup>. All data were identical to the literature.<sup>25,34</sup>

### 3.3: Methyl 2,3-di-*O*-benzyl-5-*O*-*p*-toluensulfonyl-α-*D*-Araf **5**

*p*-Toluenesulfonyl chloride (0.60 g, 3.14 mmol) was added to a stirred solution of **4** (0.50 g, 1.45 mmol), pyridine (0.96 g, 0.98 mL, 12.4 mmol) and DMAP (0.10 g, 0.81 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (5 mL) at 0 °C. The mixture was stirred at room temperature for 16 h then diluted with ethyl acetate (10 mL), the organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 100 mL). The combined organic layer were washed with water (50 mL), brine (50 mL), dried and the solvent was evaporated. The residue was purified by column chromatography eluting with hexane/ethyl acetate (4:1) affording the title compound as a colorless oil **5** (0.65 g, 90%), [α]<sub>D</sub><sup>23</sup> +55 (c 0.10, CHCl<sub>3</sub>) [lit.<sup>25</sup> [α]<sub>D</sub><sup>27</sup> +57.0 (c 0.40, CHCl<sub>3</sub>)], which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.77 (2H, d, *J* 8.2 Hz), 7.41 – 7.25 (12H, m), 4.85 (1H, s), 4.56 – 4.38 (4H, m), 4.22 – 4.15 (1H, m), 4.12 (2H, br d, *J* 4.6 Hz), 3.94 (1H, br d, *J* 2.6 Hz), 3.81 (1H, dd, *J* 5.9, 2.7 Hz), 3.33 (3H, s), 2.42 (3H, s); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 144.8, 137.3, 137.2, 132.7, 129.7, 128.4, 128.0, 127.9, 127.89, 127.8, 127.79, 107.4, 87.5, 82.8, 79.2, 72.2, 71.9, 68.8, 55.1, 21.6; ν<sub>max</sub>: 3064, 3032, 2916, 1741, 1454, 1365, 1177, 698 cm<sup>-1</sup>. All data were identical to the literature.<sup>25</sup>

### 3.4: Methyl 5-*O*-behenoyl-α-*D*-Araf **8a**

(a) A solution of N,N'-dicyclohexylcarbodiimide (0.089 g, 0.432 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1 mL) was added dropwise to a stirred solution of **4** (0.10 g, 0.29 mmol), DMAP (0.042 g, 0.343 mmol) and behenic acid (0.10 g, 0.29 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1 mL) at 0 °C under nitrogen. The mixture was stirred for 30 min. then the precipitate of dicyclohexyl urea was filtered off and washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), the solvent was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (10:1) to afford methyl 2,3-di-*O*-benzyl-5-*O*-behenoyl-α-*D*-Araf<sup>31</sup> as a colorless oil **7a** (0.15 g, 80%) [MALDI-Found (M+Na)<sup>+</sup>: 689.5, C<sub>42</sub>H<sub>66</sub>NaO<sub>6</sub> requires: 689.4], [α]<sub>D</sub><sup>24</sup> +40 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 7.41 – 7.28 (10H, m), 4.95 (1H, s), 4.59 (1H, d, *J* 12.0 Hz), 4.57 (1H, d, *J* 12 Hz), 4.52 (1H, d, *J* 12 Hz), 4.49 (1H, d, *J* 12 Hz), 4.29 (1H, dd, *J* 11.3, 2.8 Hz), 4.24 – 4.15 (2H, m), 4.01 (1H, dd, *J* 2.9, 1.1 Hz), 3.84 (1H, dd, *J* 6.3, 2.9 Hz), 3.40 (3H, s), 2.33 – 2.26 (2H, m), 1.64 – 1.52 (2H, m), 1.34 – 1.18 (36H, m), 0.89 (3H, t, *J* 6.9 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.6, 137.5, 137.4, 128.45, 128.43, 127.9, 127.8, 107.3, 88.0, 83.4, 79.4, 72.3, 72.0, 63.5, 55.0, 34.1, 31.9, 29.7, 29.6, 29.62, 29.5, 29.3, 29.27, 29.1, 24.8, 22.7, 14.1; ν<sub>max</sub>: 3034, 2915, 2849, 1741, 1471, 1100, 758 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0026 g, 0.15 fold by weight) was added to a stirred solution of **7a** (0.010 g, 0.01 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub>: MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The reaction

mixture was stirred for 24 h then filtered and the precipitate washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), the filtrate was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (1:1) to afford the title compound as a colorless oil **8a** (0.0079 g, 81%) [MALDI-Found (M+Na)<sup>+</sup>: 509.5, C<sub>28</sub>H<sub>54</sub>NaO<sub>6</sub> requires: 509.3], [α]<sub>D</sub><sup>22</sup> +46 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 4.92 (1H, s), 4.32 – 4.29 (2H, m), 4.22 (1H, dd, *J* 7.1, 3.9 Hz), 4.09 (1H, br. s), 3.90 (1H, br.s), 3.42 (3H, s), 2.81 – 2.67 (1H, m), 2.51 – 2.39 (1H, m), 2.38 – 2.32 (2H, m), 1.62 (2H, dd, *J* 14.7, 7.3 Hz), 1.35 – 1.20 (36H, m), 0.89 (3H, t, *J* 7.0 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.5, 108.8, 83.7, 79.8, 78.0, 63.8, 55.0, 34.1, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 24.8, 22.6, 14.1; ν<sub>max</sub>: br. 3500, 2917, 2850, 1739, 1464, 1100, 758 cm<sup>-1</sup>.

### 3.5: Methyl 2,3-di-*O*-benzyl-5-*O*-palmitoyl-α-*D*-Araf **8b**

(a) Cesium hydrogen carbonate (0.19 g, 1.0 mmol) was added to a stirred solution of **5** (0.1 g, 0.2 mmol) and palmitic acid (0.061 g, 0.237 mmol) in dry DMF:THF (1: 5, 5 mL) at room temperature and the reaction mixture was stirred at 70 °C for two days. The suspension was diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL), dried, filtered and evaporated to give a thick oil residue. The residue was purified by column chromatography eluting with hexane/ethyl acetate (10:1) to give methyl 2,3-di-*O*-benzyl-5-*O*-palmitoyl-α-*D*-Araf **7b** (0.089 g, 76%).<sup>25</sup> as a colorless oil [MALDI-Found (M+Na)<sup>+</sup>: 605.1, C<sub>36</sub>H<sub>54</sub>NaO<sub>6</sub> requires: 605.3], [α]<sub>D</sub><sup>22</sup> +37 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.95 (1H, s), 4.59 (1H, d, *J* 12.0 Hz), 4.57 (1H, d, *J* 11.7 Hz), 4.52 (1H, d, *J* 12.0 Hz), 4.49 (1H, d, *J* 11.8 Hz), 4.29 (1H, dd, *J* 11.3, 2.9 Hz), 4.24 – 4.15 (2H, m), 4.01 (1H, dd, *J* 3.0, 1.2 Hz), 3.84 (1H, dd, *J* 6.2, 2.9 Hz), 3.40 (3H, s), 2.32 – 2.27 (2H, m), 1.64 – 1.54 (2H, m), 1.26 (24H, s), 0.89 (3H, t, *J* 7.0 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.6, 137.5, 137.3, 128.4, 128.3, 127.9, 127.8, 107.3, 88.0, 83.4, 79.3, 72.3, 72.0, 63.5, 54.9, 34.1, 31.9, 29.7, 29.68, 29.6, 29.4, 29.32, 29.3, 29.1, 24.8, 22.7, 14.1; ν<sub>max</sub>: 3064, 3032, 2924, 2853, 1740, 1455, 1365, 1107, 735 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.01 g, 0.15 fold by weight) was added to a stirred solution of **7b** (0.06 g, 0.10 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub>: MeOH, (1:1, 2 mL) at room temperature under hydrogen atmosphere. The reaction mixture was stirred for 24 h then filtered and the precipitate was washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), the filtrate was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (1:1) to afford the title compound as a colorless oil **8b** (0.04 g, 88%),<sup>34</sup> [α]<sub>D</sub><sup>18</sup> +1.2 (c 0.10, CHCl<sub>3</sub>) [MALDI-Found (M+Na)<sup>+</sup>: 425.5, C<sub>22</sub>H<sub>42</sub>NaO<sub>6</sub> requires: 425.2] which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 4.93 (1H, s), 4.33 – 4.30 (2H, m), 4.22 (1H, dd, *J* 6.9, 3.9 Hz), 4.09 (1H, br. s), 3.91 (1H, br. s), 3.43 (3H, s), 2.35 (2H, t, *J* 7.6 Hz), 1.67 – 1.60 (2H, m), 1.56 (2H, br. s), 1.36 – 1.21 (24H, m), 0.89 (3H, t, *J* 6.9 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.5, 108.8, 83.8, 79.8, 78.0, 63.8, 55.1, 34.1, 31.9, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 24.8, 22.6, 14.1; ν<sub>max</sub>: br. 3308, 2916, 2848, 1742, 1472, 1099, 728 cm<sup>-1</sup>.

### 3.6: Methyl 5-*O*-oleoyl-α-*D*-Araf **8c**

(a) Cesium hydrogen carbonate (0.47 g, 2.42 mmol) was added to a stirred solution of **5** (0.26 g, 0.52 mmol) and oleic acid (0.10 g, 0.35 mmol) in dry DMF:THF (1:5, 2 mL) at room temperature and the mixture was stirred at 70 °C for two days. The suspension was diluted with ethyl acetate (10 mL) and water (10 mL). The

organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL). The organic layer was dried, filtered and evaporated to give a thick oil residue. This was purified by column chromatography eluting with hexane/ethyl acetate (7:2) to afford *methyl 2,3-di-O-benzyl-5-O-oleoyl-α-D-Araf* as a colorless oil **7c** (0.18 g, 83%) [MALDI-Found (M+Na)<sup>+</sup>: 631.3, C<sub>38</sub>H<sub>56</sub>NaO<sub>6</sub> requires: 631.4], [α]<sub>D</sub><sup>22</sup> +45 (c 0.10, CHCl<sub>3</sub>) which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 5.41 – 5.30 (2H, m), 4.95 (1H, s), 4.59 (1H, d, *J* 12.0 Hz), 4.57 (1H, d, *J* 12 Hz), 4.52 (1H, d, *J* 12 Hz), 4.49 (1H, d, *J* 12 Hz), 4.29 (1H, dd, *J* 11.0, 2.5 Hz), 4.24 – 4.15 (2H, m), 4.0 (1H, br d, *J* 2.1 Hz), 3.84 (1H, dd, *J* 6.2, 2.8 Hz), 3.40 (3H, s), 2.30 (2H, t, *J* 7.6 Hz), 2.06 – 1.97 (4H, m), 1.64 – 1.54 (4H, m), 1.28 (18H, br m), 0.89 (3H, t, *J* 6.8 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.6, 137.5, 137.4, 130.0, 129.7, 128.5, 128.4, 127.9, 107.3, 87.9, 83.3, 79.3, 72.3, 72.0, 63.5, 55.0, 34.0, 31.9, 29.7, 29.6, 29.5, 29.3, 29.17, 29.11, 29.1, 27.25, 27.2, 24.8, 22.6, 14.1; ν<sub>max</sub>: 3005, 3089, 3031, 2926, 2855, 1740, 1454, 1050, 735 cm<sup>-1</sup>.

(b) Boron trichloride<sup>49</sup> (0.32 mL, 1M) was added to a stirred solution of compound (**7c**) (0.02 g, 0.03 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (2 mL) at -78 °C. The mixture was stirred for 2 h then quenched with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (1:1, 2 mL) and the solvent was evaporated and the residue was purified by column chromatography eluting with hexane/ethyl acetate (1:1) to afford the title compound (**7c**) (9 mg, 64%) [Found (MALDI) (M+Na)<sup>+</sup>: 451.3, C<sub>24</sub>H<sub>44</sub>NaO<sub>6</sub> requires: 451.3], [α]<sub>D</sub><sup>18</sup> +5.0 (c 0.10, CHCl<sub>3</sub>) which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub> + few drops CD<sub>3</sub>OD): 5.35 – 5.26 (2H, m), 4.77 (1H, d, *J* 4.3 Hz), 4.24 (1H, dd, *J* 11.7, 2.1 Hz), 4.11 – 4.05 (1H, m), 4.02 – 3.97 (1H, m), 3.97 – 3.91 (2H, m), 3.39 (3H, s), 2.32 (2H, t, *J* 7.6 Hz), 2.04 – 1.92 (4H, m), 1.65 – 1.54 (2H, m), 1.35 – 1.15 (22H, m), 0.84 (3H, t, *J* 6.7 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub> + few drops CD<sub>3</sub>OD): 174.3, 129.96, 129.7, 102.3, 79.9, 77.7, 75.8, 65.4, 55.2, 49.6, 49.3, 49.1, 48.9, 48.7, 34.1, 31.8, 29.7, 29.6, 29.4, 29.2, 29.1, 29.0, 27.1, 27.09, 24.8, 22.6, 13.98; ν<sub>max</sub>: br. 3468, 2917, 2850, 1735, 1454, 1050, 824 cm<sup>-1</sup>.

### 3.7: Methyl 5-O-stearyl-α-D-Araf

Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.012 g, 0.15 fold by weight) was added to a stirred solution of **7c** (0.08 g, 0.13 mmol) in dry (CH<sub>2</sub>Cl<sub>2</sub> : MeOH, 1:1, 3 mL) at room temperature under hydrogen atmosphere. The mixture was stirred for 24 h then filtered and the precipitate washed with CH<sub>2</sub>Cl<sub>2</sub> (15 mL), the filtrate was evaporated and the residue was purified by column chromatography eluting with hexane / ethyl acetate (1:1) to afford the title compound as a colorless oil (0.045 g, 80%) [MALDI-Found (M+Na)<sup>+</sup>: 453.0, C<sub>24</sub>H<sub>46</sub>NaO<sub>6</sub> requires: 453.3]; [α]<sub>D</sub><sup>18</sup> +2 (c 0.1, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 4.92 (1H, s), 4.30 (2H, d, *J* 4.0 Hz), 4.21 (1H, dd, *J* 7.3, 3.8 Hz), 4.17 – 4.06 (1H, m), 3.90 (1H, br d, *J* 10.1 Hz), 3.42 (3H, s), 2.77 (1H, d, *J* 10.2 Hz), 2.52 (1H, d, *J* 7.9 Hz), 2.35 (2H, t, *J* 7.6 Hz), 1.69 – 1.58 (2H, m), 1.34 – 1.21 (28H, m), 0.88 (3H, t, *J* 6.8 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 173.5, 108.8, 83.7, 79.8, 78.0, 63.8, 55.1, 50.8, 34.1, 31.9, 29.7, 29.6, 29.6, 29.4, 29.3, 29.2, 29.05, 24.79, 22.67, 14.10; ν<sub>max</sub>: br. 3468, 2917, 2850, 1735, 1454, 1050, 824 cm<sup>-1</sup>.

### 3.8: Methyl 5-O-(2-((R)-1-hydroxy-18-[(1S, 2R)-2-[(17S, 18S)-17-methoxy-18-methylhexatriacontyl]cyclopropyl]octadecyl]tetracosanoate) α-D-Araf 8d

(a) Cesium hydrogen carbonate (0.056 g, 0.288 mmol) was added to a solution of **5** (0.031 g, 0.062 mmol) and (R)-2-((R)-1-hydroxy-18-[(1S,2R)-2-((17S,18S)-17-methoxy-18-methylhexatriacontyl) cyclopropyl]octadecyl]tetracosanoic acid (0.051 g, 0.041 mmol)<sup>37</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and the mixture was stirred at 70 °C for two days. Work up as before gave *methyl 2,3-di-O-benzyl-5-O-(2-((R)-1-hydroxy-18-[(1S,2R)-2-[(17S,18S)-17-methoxy-18-methylhexatriacontyl]cyclopropyl]octadecyl]-tetracosanoate) α-D-Araf* **7d** as a thick colorless oil (0.05 g, 77%), [Found (M+Na)<sup>+</sup>: 1574.4044, C<sub>103</sub>H<sub>186</sub>NaO<sub>8</sub> requires: 1574.4040]; [α]<sub>D</sub><sup>20</sup> +23 (c 0.10, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12.0 Hz), 4.56 (1H, d, *J* 12 Hz), 4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.33 – 4.27 (2H, m), 4.25 – 4.19 (1H, m), 3.99 (1H, br d, *J* 2.0 Hz), 3.84 (1H, dd, *J* 6.4, 2.6 Hz), 3.67 – 3.59 (1H, m), 3.37 (3H, s), 3.35 (3H, s), 2.99 – 2.94 (1H, m), 2.52 (1H, d, *J* 8.3 Hz), 2.43 (1H, dt, *J* 9.4, 5.4 Hz), 1.72 – 1.61 (2H, m), 1.59 – 1.03 (141H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H, d, *J* 6.9 Hz), 0.70 – 0.61 (2H, m), 0.60 – 0.53 (1H, dt, *J* 4, 8 Hz), -0.33 (1H, br. q *J* 5.1 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 175.0, 128.5, 128.4, 127.9, 127.8, 107.2, 87.9, 85.4, 83.7, 79.4, 72.4, 72.1, 63.5, 57.7, 54.9, 51.53, 35.52, 35.3, 32.36, 31.9, 30.5, 30.48, 30.4, 30.3, 30.23, 30.12, 30.0, 29.98, 29.94, 29.9, 29.86, 29.84, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.26, 29.2, 29.19, 29.1, 29.0, 29.04, 28.7, 28.67, 27.5, 27.4, 26.1, 25.7, 22.7, 22.6, 15.7, 14.8, 14.1, 10.9; ν<sub>max</sub>: 3479, 3064, 2924, 2853, 1735, 1494, 1455, 1100 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0033 g, 0.15 fold by weight) was added to a stirred solution of **7d** (0.022 g, 0.014 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight; work up as before gave the title compound as a thick colorless oil **8d** (0.016 g, 79%) [Found (M+NH<sub>4</sub>)<sup>+</sup>: 1389.3550, C<sub>89</sub>H<sub>178</sub>NO<sub>8</sub> requires: 1389.3552]; [α]<sub>D</sub><sup>21</sup> +28 (c 0.10, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.49 (1H, dd, *J* 12, 4.0 Hz), 4.35 (1H, dd, *J* 12, 4.1 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br.s), 3.98 (1H, br.s), 3.77 – 3.66 (1H, m), 3.41 (3H, s), 3.35 (3 H, s), 3.01 – 2.92 (1H, m), 2.85 (1H, s), 2.54 – 2.36 (1H, ddd, *J* 10.1, 6.9, 5.1 Hz), 1.48 – 1.17 (144H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H, d, *J* 6.9 Hz), 0.70 – 0.61 (2H, m), 0.61 – 0.52 (1H, dt, *J* 7.9, 3.9 Hz), -0.33 (1H, br. q *J* 5.1 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 174.9, 108.7, 85.4, 83.6, 80.5, 78.4, 72.8, 63.2, 57.7, 54.9, 52.3, 35.3, 35.2, 32.3, 31.9, 30.7, 30.69, 30.64, 30.62, 30.55, 30.5, 30.4, 30.37, 30.3, 30.28, 30.2, 30.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.57, 29.55, 29.5, 29.4, 29.3, 29.28, 29.25, 29.2, 29.1, 29.0, 29.03, 29.0, 28.9, 28.7, 28.6, 28.5, 28.3, 27.5, 27.4, 26.1, 25.4, 22.6, 15.7, 14.8, 14.1, 10.9; ν<sub>max</sub>: br. 3435, 2918, 2850, 1732, 1455, 1100 cm<sup>-1</sup>.

### 3.9: Methyl 5-O-(2-((R)-1-hydroxy-18-[(1R, 2S)-2-[(17S,18S)-17-methoxy-18-methylhexatriacontyl]cyclo propyl]octadecyl]-tetracosanoate) α-D-Araf 8e

(a) Cesium hydrogen carbonate (0.098 g, 0.505 mmol) was added to a stirred solution of **5** (0.0546 g, 0.1000 mmol) and (R)-2-((R)-1-hydroxy-18-[(1R,2S)-2-((17S,18S)-17-methoxy-18-methyl-hexatriacontyl)cyclopropyl]octadecyl]tetracosanoic acid (0.100 g, 0.081 mmol)<sup>37</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and the reaction mixture was stirred at 70 °C for two days. The suspension was diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (2 × 10 mL). The combined organic layers were washed with water (15 mL) and brine (15 mL), then dried, filtered and evaporated to give a thick



oil residue. The residue was purified by column chromatography eluting with hexane/ethyl acetate (10:1) to give *methyl 2,3-di-O-benzyl-5-O-(2-[(R)-1-hydroxy-18-[(1R,2S)-2-[(17S,18S)-17-methoxy-18-methyl-hexatriacontyl]-cyclopropyl]octadecyl]tetracosanoate)α-D-Araf* as a colorless thick oil **7e** (0.095 g, 75%) [Found (M+Na)<sup>+</sup>: 1574.4085, C<sub>103</sub>H<sub>186</sub>NaO<sub>8</sub> requires: 1574.4040], [α]<sub>D</sub><sup>23</sup> +18 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 7.38 – 7.29 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12 Hz), 4.56 (1H, d, *J* 12 Hz), 4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.31 – 4.28 (2H, m), 4.24 – 4.20 (1H, m), 3.99 (1H, dd, *J* 2.7, 0.9 Hz), 3.84 (1H, dd, *J* 6.4, 2.6 Hz), 3.66 – 3.60 (1H, m), 3.38 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.52 (1H, br. s), 2.43 (1H, dt, *J* 9.1, 5.5 Hz), 1.72 – 1.61 (2H, m), 1.60 – 1.07 (141H, m), 0.89 (6H, t, *J* 6.9 Hz), 0.86 (3H, d, *J* 6.8 Hz), 0.69 – 0.62 (2H, m), 0.57 (1H, dt, *J* 8.4, 4.1 Hz), – 0.32 (1H, q, *J* 5.2 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 175.0, 137.5, 137.3, 128.6, 128.5, 127.9, 127.8, 107.2, 87.9, 85.4, 83.7, 79.4, 72.4, 72.2, 72.1, 63.5, 57.7, 54.9, 51.5, 35.5, 35.3, 32.5, 32.3, 31.9, 31.3, 31.0, 30.9, 30.8, 30.79, 30.6, 30.58, 30.5, 30.47, 30.4, 30.38, 30.37, 30.3, 30.29, 30.2, 30.19, 30.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.18, 29.17, 29.16, 29.15, 29.0, 28.9, 28.8, 28.7, 28.5, 28.4, 28.3, 27.6, 27.4, 27.3, 26.9, 26.1, 25.7, 22.6, 15.7, 14.8, 14.1, 10.9; ν<sub>max</sub>: 3479, 3064, 2923, 2853, 1733, 1465, 1100, 721 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.003 g, 0.15 fold by weight) was added to a stirred solution of **7e** (0.020 g, 0.012 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then filtered and the solvent was evaporated to give a residue; column chromatography eluting with hexane/ethyl acetate (1:1) gave the title compound as a colorless oil **8e** (0.011 g, 65%) [Found (M+Na)<sup>+</sup>: 1394.3138, C<sub>89</sub>H<sub>174</sub>NaO<sub>8</sub> requires: 1394.3101], [α]<sub>D</sub><sup>16</sup> +10 (c 0.70, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.50 (1H, dd, *J* 12, 3.8 Hz), 4.32 (1H, dd, *J* 12, 4.4 Hz), 4.20 – 4.16 (1H, m), 4.07 (1H, d, *J* 5.6 Hz), 4.00 – 3.96 (1H, m), 3.74 – 3.64 (1H, m), 3.41 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.79 – 2.7 (2H, m), 2.48 – 2.39 (1H, m), 2.39 – 2.32 (1H, m), 1.6 – 1.05 (143H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H, d, *J* 6.9 Hz), 0.69 – 0.63 (2H, m), 0.60 – 0.53 (1H, dt, *J* ), – 0.32 (1H, br. q, *J* 4.5 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 174.9, 108.7, 85.4, 83.8, 80.4, 78.4, 72.8, 63.2, 57.7, 55.0, 52.2, 35.3, 35.2, 32.3, 31.9, 30.5, 30.2, 30.0, 29.9, 29.7, 29.68, 29.6, 29.5, 29.4, 29.3, 28.7, 27.5, 27.4, 26.1, 25.4, 22.7, 15.7, 14.99, 14.1, 10.9; ν<sub>max</sub>: br. 3436, 2918, 2850, 1732, 1467, 1099, 720 cm<sup>-1</sup>.

### 3.10: Methyl 5-O-(2-[(R)-1-hydroxy-18-[(1R,2S)-2-[(17S,18S)-17-methoxy-18-methylhexatriacontyl]cyclopropyl]octadecyl] hexacosanoate) α-D-Araf **8f**

(a) Cesium hydrogen carbonate (0.083 g, 0.428 mmol) was added with stirring to **5** (0.0457 g, 0.091 mmol) and (R)-2-[(R)-1-hydroxy-18-[(1R,2S)-2-[(17S,18S)-17-methoxy-18-methylhexatriacontyl]cyclopropyl]octadecyl]hexacosanoic acid (0.076 g, 0.060 mmol)<sup>40</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and the mixture was stirred at 70 °C for two days then worked up as before to give *methyl 2,3-di-O-benzyl-5-O-(2-[(R)-1-hydroxy-18-[(1R,2S)-2-[(17S,18S)-17-methoxy-18-methylhexatriacontyl]-cyclopropyl]octadecyl]hexacosanoate)α-D-Araf* **7f** (0.077 g, 80%) as a thick colorless oil [Found (M+Na)<sup>+</sup>: 1602.4304, C<sub>105</sub>H<sub>190</sub>NaO<sub>8</sub> requires: 1602.4353], [α]<sub>D</sub><sup>22</sup> +18 (c 0.10, CHCl<sub>3</sub>), which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12 Hz), 4.56 (1H, d, *J* 12 Hz), 4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.31 – 4.28 (2H, m), 4.26 – 4.19 (1H, m), 4.40 (1H, dd, *J* 5.6, 2.5 Hz), 3.84 (1H, dd, *J* 6.3, 2.5 Hz), 3.68 – 3.58 (1H, m), 3.37 (3H, s),

3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.50 (1H, d, *J* 8.2 Hz), 2.44 (1H, dt, *J* 8.7, 5.4 Hz), 1.75 – 1.56 (2H, m), 1.58 – 1.04 (145H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.86 (3H, d, *J* 6.9 Hz), 0.70 – 0.62 (2H, m), 0.57 (1H, dt, *J* 8.4 Hz), – 0.32 (1H, br. q, *J* 5.0 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 175.0, 137.4, 137.3, 128.5, 128.4, 128.0, 127.9, 127.89, 127.88, 127.87, 127.86, 107.2, 87.8, 85.4, 83.7, 79.4, 72.4, 72.2, 72.1, 63.4, 57.7, 54.9, 51.5, 35.5, 35.3, 32.4, 31.9, 30.8, 30.7, 30.67, 30.6, 30.55, 30.5, 30.45, 30.4, 30.38, 30.37, 30.36, 30.2, 30.17, 30.16, 30.15, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.27, 29.26, 29.2, 29.14, 29.1, 29.07, 29.02, 29.01, 28.94, 28.9, 28.7, 28.65, 28.6, 28.52, 28.5, 27.5, 27.4, 26.1, 25.7, 22.6, 15.7, 14.8, 14.1, 10.9; ν<sub>max</sub>: br. 3522, 3064, 2921, 2851, 1723, 1467, 1027, 732 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.003 g, 0.15 fold by weight) was added to a stirred solution of **7f** (0.020 g, 0.012 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then worked up as before to give the title compound as a thick colorless oil **8f** (0.014 g, 76%) [Found (M+Na)<sup>+</sup>: 1422.3425, C<sub>91</sub>H<sub>178</sub>NaO<sub>8</sub> requires: 1422.3414]; [α]<sub>D</sub><sup>21</sup> +10 (c 0.10, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.51 (1H, dd, *J* 12, 3.9 Hz), 4.33 (1H, dd, *J* 12.0, 4.1 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br. s), 4.01 – 3.95 (1H, m), 3.75 (1H, d, *J* 5.3 Hz), 3.73 – 3.66 (1H, m), 3.41 (3H, s), 3.35 (3H, s), 3.00 – 2.93 (1H, m), 2.85 – 2.70 (2H, m), 2.50 – 2.39 (1H, m), 1.72 – 1.61 (2H, m), 1.60 – 1.03 (145H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.85 (3H, d, *J* 6.8 Hz), 0.70 – 0.61 (2H, m), 0.60 – 0.53 (1H, dt, *J* 8, 4 Hz), – 0.33 (1H, br. q, *J* 5.2 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 175.0, 108.7, 85.5, 83.8, 80.4, 78.4, 72.8, 63.2, 57.7, 55.0, 52.2, 35.3, 35.2, 32.3, 31.9, 30.5, 30.2, 29.9, 29.89, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 28.7, 27.6, 27.4, 26.1, 22.4, 22.7, 15.77, 14.8, 14.1, 10.9; ν<sub>max</sub>: br. 3436, 2921, 2852, 1732, 1493, 1455, 759 cm<sup>-1</sup>.

### 3.11: Methyl 5-O-(2-[(1R)-1-hydroxy-16-[(1R,2S)-2-[20-methyl-19-oxooctatriacontyl]cyclopropyl] hexadecyl] tetra-cosanoate) α-D-Araf **8g**

(a) Cesium hydrogen carbonate (0.086 g, 0.443 mmol) was added to a stirred solution of **5** (0.0475 g, 0.0953 mmol) and (R)-2-[(R)-1-hydroxy-16-[(1R,2S)-2-[(S)-20-methyl-19-oxo-octatriacontyl]-cyclopropyl]hexadecyl]tetracosanoic acid (0.077 g, 0.063 mmol)<sup>41</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and stirred at 70 °C for two days. Work up as before gave *methyl 2,3-di-O-benzyl-5-O-(2-[(1R)-1-hydroxy-16-[(1R,2S)-2-[20-methyl-19-oxooctatriacontyl]cyclopropyl]hexadecyl]tetracosanoate) α-D-Araf* as a thick colorless oil **7g** (0.077 g, 80%) [Found (M+Na)<sup>+</sup>: 1558.3708, C<sub>102</sub>H<sub>182</sub>NaO<sub>8</sub> requires: 1558.3727], [α]<sub>D</sub><sup>21</sup> +21 (c 0.10, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12.0 Hz), 4.56 (1H, d, *J* 12 Hz), 4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.32 – 4.28 (2H, m), 4.25 – 4.19 (1H, m), 3.99 (1H, br. d, *J* 2.2 Hz), 3.84 (1H, dd, *J* 6.4, 2.8 Hz), 3.67 – 3.59 (1H, m), 3.38 (3H, s), 2.57 – 2.47 (2H, m), 2.47 – 2.38 (3H, m), 1.75 – 1.09 (140H, m), 1.06 (3H, d, *J* 6.9 Hz), 0.89 (6H, t, *J* 6.7 Hz), 0.70 – 0.62 (2H, m), 0.61 – 0.53 (1H, dt, *J* 3.9, 7.9 Hz), – 0.32 (1H, br. q, *J* 5.2 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 215.2, 175.0, 137.5, 137.3, 128.5, 128.4, 127.9, 127.9, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.1, 63.4, 54.9, 51.5, 46.3, 41.1, 33.0, 31.9, 30.2, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 28.7, 27.4, 27.3, 25.7, 23.7, 22.7, 16.3, 15.7, 14.1, 10.9; ν<sub>max</sub>: br. 3524, 3030, 3063, 2922, 2851, 1732, 1715, 1465, 1107, 733 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0015 g, 0.15 fold by weight) was added to a stirred solution of **7g** (0.010 g, 0.006 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The reaction

mixture was stirred overnight then worked up as before to afford the title compound as a thick white oil **8g** (0.0069 g, 63%) [Found (M+Na)<sup>+</sup>: 1378.2788, C<sub>88</sub>H<sub>170</sub>NaO<sub>8</sub> requires: 1378.2803], [α]<sub>D</sub><sup>21</sup> +10.5 (c 0.10, CHCl<sub>3</sub> + few drops of CD<sub>3</sub>OD); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.51 (1H, dd, *J* 12, 4.0 Hz), 4.33 (1H, dd, *J* 12.0, 4.1 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br. s), 4.02 – 3.95 (1H, m), 3.74 – 3.66 (1H, m), 3.41 (3H, s), 2.90 – 2.71 (2H, m), 2.56 – 2.48 (1H, m), 2.48 – 2.37 (4H, m, including the OH), 2.23 – 1.10 (140H, m), 1.05 (3H, d, *J* 6.9 Hz), 0.89 (6H, t, *J* 6.8 Hz), 0.70 – 0.61 (2H, m), 0.59 – 0.53 (1H, dt, *J* 7.9, 3.9 Hz), -0.33 (1H, br. q, *J* 5.2 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub> + few drops of CD<sub>3</sub>OD): 215.0, 174.9, 108.7, 83.8, 80.4, 78.4, 72.8, 63.2, 54.9, 52.1, 46.3, 41.1, 35.2, 33.0, 31.9, 30.2, 29.7, 29.6, 29.5, 29.45, 29.4, 29.35, 29.3, 28.7, 27.4, 27.3, 25.4, 23.7, 22.7, 16.36, 15.7, 14.1, 10.9; ν<sub>max</sub>: br. 3436, 2918, 2850, 1731, 1708, 1467, 1170 cm<sup>-1</sup>.

### 3.12: Methyl 5-*O*-(2-[(1*R*)-1-hydroxy-16-[(1*R*,2*S*)-2-(20-methyl-19-oxo-octatriacontyl)cyclopropyl]hexadecyl]-hexacosanoate)-α-*D*-Araf **8g'**

(a) Cesium hydrogen carbonate (0.062g, 0.319 mmol) was added to a stirred solution of **5** (0.048 g, 0.096 mmol) and (*R*)-2-[(*R*)-1-hydroxy-16-[(1*R*,2*S*)-2-[(*S*)-20-methyl-19-oxo-octatriacontyl]-cyclopropyl]hexadecyl]hexacosanoic acid (0.080 g, 0.064 mmol)<sup>42</sup> in dry DMF : THF (1:5, 4 mL) at room temperature and the mixture was stirred at 70 °C for 2 days, then worked up and purified as before to afford methyl 2,3-*di-O*-benzyl-5-*O*-(2-[(1*R*)-1-hydroxy-16-[(1*R*,2*S*)-2-(20-methyl-19-oxooctatriacontyl)-cyclopropyl]hexadecyl]hexacosanoate)-α-*D*-Araf as a colourless thick oil **7g'** (72 mg, 71%) [Found (M+NH<sub>4</sub>)<sup>+</sup>: 1581.4467, C<sub>104</sub>H<sub>190</sub>NO<sub>8</sub> requires: 1581.4486], [α]<sub>D</sub><sup>23</sup> + 25 (c 0.10, CHCl<sub>3</sub>) which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.42 – 7.27 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12.0 Hz), 4.56 (1H, d, *J* 11.8 Hz), 4.50 (1H, d, *J* 12.0 Hz), 4.47 (1H, d, *J* 11.8 Hz), 4.34 – 4.26 (2H, m), 4.22 (1H, dt, *J* 4.6, 9.1 Hz), 3.99 (1H, br. d, *J* 2.1 Hz), 3.84 (1H, dd, *J* 2.6, 6.4 Hz), 3.68 – 3.58 (1H, m), 3.38 (3H, s), 2.56 – 2.47 (2H, m, including OH), 2.47 – 2.37 (3H, m), 1.76 – 1.09 (144H, m), 1.05 (3H, d, *J* 6.9 Hz), 0.89 (6H, t, *J* 6.8 Hz), 0.70 – 0.61 (2H, m), 0.56 (1H, dt, *J* 4.0, 8.0 Hz), -0.33 (1H, br. q, *J* 5.2 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 215.2, 175.0, 137.4, 137.2, 128.5, 128.4, 128.0, 127.9, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.1, 72.0, 63.4, 54.9, 51.5, 46.3, 41.1, 35.5, 33.0, 31.9, 30.2, 29.7, 29.67, 29.6, 29.57, 29.5, 29.46, 29.4, 29.3, 28.7, 27.4, 27.3, 25.8, 23.7, 22.7, 16.4, 15.8, 14.1, 10.9; ν<sub>max</sub>: 3457, 3064, 3032, 2921, 2851, 1717, 1678, 1466, 1101, 755, 697 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.01g, 0.15 fold by weight) was added to a stirred solution of compound **7g'** (0.066 g, 0.042 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was worked up and purified as before to afford the title compound **8g'** as a colourless thick oil (39 mg, 66%) [Found (M+NH<sub>4</sub>)<sup>+</sup>: 1401.3546, C<sub>90</sub>H<sub>178</sub>NO<sub>8</sub>, requires: 1401.3547], [α]<sub>D</sub><sup>23</sup> +11 (c 0.10, CHCl<sub>3</sub>) which showed δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub> + few drops CD<sub>3</sub>OD): 4.89 (1H, s), 4.51 (1H, dd, *J* 3.9, 11.9 Hz), 4.32 (1H, dd, *J* 4.0, 11.9 Hz), 4.21 – 4.15 (1H, m), 4.07 (1H, br. s), 3.98 (1H, br. d, *J* 1.8 Hz), 3.74 – 3.66 (1H, m), 3.41 (3H, s), 2.87 – 2.67 (2H, m), 2.55 – 2.38 (4H, m), 1.71 – 1.12 (145H, m), 1.05 (3H, d, *J* 6.9 Hz), 0.89 (6H, t, *J* 6.8 Hz), 0.69 – 0.61 (2H, m), 0.56 (1H, dt, *J* 4.0, 8.0 Hz), -0.33 (1H, br. q, *J* 5.2 Hz); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub> + few drops CD<sub>3</sub>OD): 215.4, 175.0, 108.7, 83.6, 80.6, 78.4, 72.8, 63.2, 55.0, 52.3, 50.9, 46.3, 41.1, 35.2, 33.0, 31.9, 30.2, 29.7, 29.65, 29.6, 29.5, 29.45, 29.41, 29.40, 29.3, 28.7, 27.4, 27.3, 25.4, 23.7, 22.7, 16.4, 15.8, 14.1, 10.9; ν<sub>max</sub>: 3405, 2920, 2851, 1713, 1712, 1466, 1108, 759 cm<sup>-1</sup>.

### 3.13: Methyl 5-*O*-(2-[(1*R*)-1-hydroxy-17-[(1*S*, 2*R*)-2-[(2*S*)-22-methyl-21-oxotetracontan-2-yl]cyclopropyl]heptadecyl]hexa- cosanoate) α-*D*-Araf **8h**

(b) Cesium hydrogen carbonate (0.045 g, 0.232 mmol) was added with stirring to **5** (0.025 g, 0.050mmol) and (*R*)-2-[(*R*)-17-[(1*S*,2*R*)-2-[(1*S*)-1,21-dimethyl-20-oxo-nonatriacontyl]cyclopropyl]-1-hydroxy heptadecyl]hexacosanoic acid methyl ester (0.043 g, 0.033 mmol)<sup>43</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and stirred at 70 °C for two days, then worked up as before to give methyl 2,3-*di-O*-benzyl-5-*O*-(2-[(1*R*)-1-hydroxy-17-[(1*S*,2*R*)-2-[(2*S*)-22-methyl-21-oxotetra-contan-2-yl]cyclopropyl]heptadecyl]hexacosanoate) α-*D*- Araf **7h** as a thick colorless oil (0.050 g, 92%) [Found (M+Na)<sup>+</sup>:1628.4539, C<sub>107</sub>H<sub>192</sub>NaO<sub>8</sub> requires: 1628.4509], [α]<sub>D</sub><sup>21</sup> +16.8 (c 0.1, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.40 – 7.27 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12.0 Hz), 4.56 (1H, d, *J* 12 Hz), 4.53 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.33 – 4.28 (2H, m), 4.22 (1H, dt, *J* 10.7, 4.6 Hz), 3.99 (1 H, br d, *J* 1.9 Hz), 3.84 (1H, dd, *J* 6.4, 2.6 Hz), 3.68 – 3.58 (1H, dt, *J* 7.6, 5.2 Hz), 3.38 (3H, s), 2.58 – 2.47 (2H, m), 2.47 – 2.38 (3H, m), 1.72 – 1.13 (146H, m), 1.06 (3H, d, *J* 6.9 Hz), 0.89 (9H, including a triplet *J* 7.5 Hz), 0.72 – 0.61 (1H, m), 0.50 – 0.41 (1H, m), 0.24 – 0.08 (3H, m); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 215.2, 175.0, 137.5, 137.3, 128.5, 128.4, 128.0, 127.9, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.1, 63.5, 54.9, 51.5, 46.3, 41.1, 38.1, 37.4, 35.5, 34.5, 33.0, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 27.4, 27.35, 27.3, 26.1, 25.7, 23.7, 22.7, 19.7, 18.6, 16.3, 14.1, 10.5; ν<sub>max</sub>: br. 3524, 3064, 3031, 2923, 2853, 1737, 1715, 1465, 1107, 734 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0015 g, 0.15 fold by weight) was added to a stirred solution of **7h** (0.010 g, 0.006 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then worked up as before to afford the title compound as a thick colorless oil **8h** (0.010 g, 80%) [Found (M+Na)<sup>+</sup>: 1448.3585, C<sub>93</sub>H<sub>180</sub>NaO<sub>8</sub> requires: 1448.3570], [α]<sub>D</sub><sup>21</sup> + 13 (c 0.10, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 4.89 (1H, s), 4.49 (1H, dd, *J* 12, 4 Hz), 4.33 (1H, dd, *J* 12, 4.2 Hz), 4.21 – 4.16 (1H, m), 4.09 (1H, d, *J* 6.8 Hz), 4.01 – 3.95 (1H, m), 3.75 (1H, d, *J* 5.4 Hz), 3.73 – 3.65 (1H, m), 3.41 (3H, s), 2.61 – 2.47 (1H, m), 2.46 – 2.28 (5H, m), 1.72 – 1.15 (146H, m), 1.06 (3H, d, *J* 6.9 Hz), 0.89 (9H, including a triplet at 0.89 with *J* 7.4 Hz), 0.76 – 0.61 (1H, m), 0.51 – 0.39 (1H, m), 0.26 – 0.05 (3H, m); δ<sub>C</sub> (101 MHz, CDCl<sub>3</sub>): 215.3, 174.9, 108.7, 83.8, 80.4, 78.8, 78.4, 72.8, 54.9, 52.2, 46.3, 44.5, 41.1, 38.1, 37.4, 35.2, 34.5, 33.0, 31.9, 30.0, 29.7, 29.6, 29.5, 29.3, 27.3, 26.1, 25.4, 23.71, 22.7, 19.7, 18.6, 16.3, 14.1, 10.5; ν<sub>max</sub>: br. 3467, 2917, 2849, 1731, 1713, 1467, 1050,720 cm<sup>-1</sup>.

### 3.14: Methyl 5-*O*-(2-[(*R*)-1-hydroxy-12-[(1*S*, 2*R*)-2-[14-[(1*S*, 2*R*)-2-eicosylcyclopropyl]tetradecyl]cyclopropyl]dodecyl]-hexacosanoate) α-*D*-Araf **8i**

(a) Cesium hydrogen carbonate (0.089g, 0.46 mmol) was added to a stirred solution of **5** (0.049 g, 0.098 mmol) and (*R*)-2-[(*R*)-1-hydroxy-12-[(1*S*,2*R*)-2-[14-[(1*S*,2*R*)-2-eicosylcyclopropyl]tetradecyl]cyclopropyl]dodecyl]hexacosanoic acid (0.075 g, 0.065 mmol)<sup>37</sup> in dry DMF:THF (1:5, 2 mL) at room temperature and then stirred at 70 °C for two days. Work up as before gave methyl 2,3-*di-O*-benzyl-5-*O*-(2-[(*R*)-1-hydroxy-12-[(1*S*,2*R*)-2-[14-[(1*S*,2*R*)-2-eicosylcyclopropyl]tetradecyl]cyclopropyl]dodecyl]hexacosanoate) α-*D*-Araf **7i** as a thick colorless oil (0.084 g, 87%) [Found (M+Na)<sup>+</sup>: 1486.3173, C<sub>98</sub>H<sub>174</sub>NaO<sub>7</sub> requires: 1486.3152], [α]<sub>D</sub><sup>21</sup> +31 (c 0.10, CHCl<sub>3</sub>); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.39 – 7.28 (10H, m), 4.92 (1H, s), 4.58 (1H, d, *J* 12 Hz), 4.56 (1H, d, *J* 12 Hz),

4.51 (1H, d, *J* 12 Hz), 4.48 (1H, d, *J* 12 Hz), 4.32 – 4.28 (2H, m), 4.25 – 4.19 (1H, m), 3.99 (1H, br d, *J* 2.3 Hz), 3.84 (1H, dd, *J* 6.4, 2.6 Hz), 3.68 – 3.59 (1H, m), 3.38 (3H, s), 2.52 (1H, d, *J* 8.3 Hz), 2.44 (1H, dt, *J* 9.3, 5.5 Hz), 1.62 – 1.03 (134H, m), 0.89 (6H, t, *J* 6.7 Hz), 0.72 – 0.62 (4H, m), 0.61 – 0.52 (2H, dt *J* 8, 4 Hz), -0.32 (2H, br. q *J* 5.1 Hz);  $\delta_{\text{C}}$  (101 MHz, CDCl<sub>3</sub>): 175.0, 137.4, 137.2, 128.5, 128.4, 128.0, 127.9, 127.89, 127.8, 107.2, 87.8, 83.7, 79.4, 72.4, 72.2, 72.1, 63.5, 54.9, 51.5, 35.5, 31.9, 30.55, 30.5, 30.45, 30.44, 30.4, 30.35, 30.3, 30.28, 30.2, 30.18, 30.1, 30.0, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.29, 29.2, 29.1, 29.03, 29.0, 28.7, 28.6, 27.4, 25.7, 22.68, 22.6, 15.7, 14.1, 10.9;  $\nu_{\text{max}}$ : 3479, 3065, 2989, 2919, 2849, 1733, 1607, 1494, 718 cm<sup>-1</sup>.

(b) Palladium hydroxide on activated charcoal (20% Pd(OH)<sub>2</sub>-C, 0.0087 g, 0.15 fold by weight) was added to a stirred solution of **7i** (0.058g, 0.039 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> : MeOH (1:1, 2 mL) at room temperature under hydrogen atmosphere. The mixture was stirred overnight then worked up as before to afford the title compound as a thick colourless oil **8i** (0.040 g, 80%) [Found (M+Na)<sup>+</sup>: 1306.2201, C<sub>84</sub>H<sub>162</sub>NaO<sub>7</sub> requires: 1306.2213], [ $\alpha$ ]<sub>D</sub><sup>23</sup> + 12 (c 0.10, CHCl<sub>3</sub>);  $\delta_{\text{H}}$  (400 MHz, CDCl<sub>3</sub> + few drops of CD<sub>3</sub>OD): 4.89 (1H, s), 4.47 (1H, dd, *J* 11.8, 4.2 Hz), 4.37 (1H, dd, *J* 11.9, 4.1 Hz), 4.20 – 4.14 (1H, m), 4.07 (1H, br. s), 3.98 (1H, br. s), 3.79 – 3.67 (1H, m), 3.40 (3H, s), 3.06 (2H, br. s), 2.45 (1H, dt, *J* 10.1, 5.0 Hz), 1.90 – 1.83 (1H, m), 1.63 – 1.07 (134H, m), 0.89 (6H, t, *J* 6.8 Hz), 0.71 – 0.61 (4H, m), 0.60 – 0.49 (2H, dt, *J* 8, 4 Hz), -0.33 (2H, br. q *J* 5.2 Hz);  $\delta_{\text{C}}$  (101 MHz, CDCl<sub>3</sub>): 175.0, 108.7, 83.4, 80.7, 78.4, 72.8, 63.2, 54.9, 52.4, 35.1, 31.9, 30.4, 30.2, 30.18, 29.7, 29.68, 29.6, 29.5, 29.48, 29.4, 29.35, 29.3, 28.7, 27.4, 25.4, 22.6, 15.7, 14.1, 10.9;  $\nu_{\text{max}}$ : 3436, 390, 2918, 2850, 1733, 1467, 1455, 1050 cm<sup>-1</sup>.

### 3.15: The ELISA assay

Serum samples were obtained from the TDR TB Specimen Bank.<sup>47</sup>

ELISA were carried out on 96-well flat-bottomed polystyrene micro-plates. Antigens were dissolved in hexane to give an antigen solution of concentration 15 µg/ml. 50 µl of this solution was added to each well, and the solvent was left to evaporate at room temperature. Control wells were coated with hexane (50 µl / well) only. Blocking was done by adding 400 µl of 0.5 % casein/PBS buffer (pH 7.4) to each well, and the plates were incubated at 25 °C for 30 min. The buffer was aspirated and any excess buffer was flicked out until the plates were dry. Serum (1 in 80 dilution in casein/PBS buffer) (50 µl / well) was added and incubated at 25 °C for 1 h. The plates were washed with 400 µl casein/PBS buffer 3 times using an automatic washer, and any excess buffer was flicked out onto a paper towel until dry. Secondary antibody (anti-human IgG (Fc specific) peroxidase conjugated antibody produced in goat (Aldrich) (diluted to a concentration of 1:2000 in casein/PBS buffer) (50 µl / well) was added, and incubated at 25 °C for 30 minutes. The plates were again washed 3 times with 400 µl casein/PBS buffer using an automatic washer, and any excess buffer was again flicked out. OPD substrate (50 µl / well) (*o*-phenylenediamine (1 mg / ml) and H<sub>2</sub>O<sub>2</sub> (0.8 mg / ml) in 0.1 M citrate buffer) was then added, and the plates were incubated for a further 30 min at 25 °C. The colour reaction was terminated by adding 2.5 M H<sub>2</sub>SO<sub>4</sub> (50 µl / well), and the absorbance was read at 492nm.

### Acknowledgments

MM wishes to thank the Ministry of Higher Education & Scientific Research - Kurdistan Region, Iraq for the award of a grant. We thank the EPSRC UK National Mass Spectrometry Facility at Swansea University for carrying out accurate mass measurements. We thank Dr A Ramsay and the Special Programme for Research and Training in Tropical Diseases at the World Health Organization for access to materials from the TDR TB Specimen Bank

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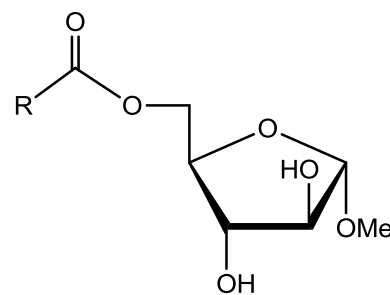
# Graphical Abstract

## Arabino Mycolates from Synthetic Mycolic Acids

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R = synthetic mycolic acid



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